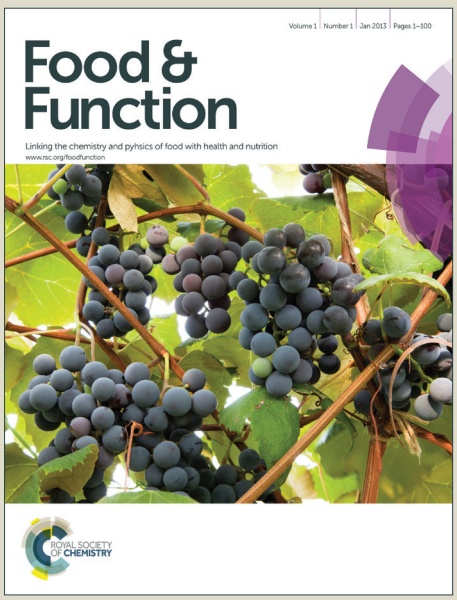
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Green tea extract improves the oxidative state of liver and brain in rats with adjuvant-induced arthritis

Geferson de Almeida Gonçalves[1,], Anacharis Babeto de Sá-Nakanishi[1,], Mariana Marques Nogueira Wendt[1], Jurandir Fernando Comar[1], Ciomar Aparecida Bersani Amado[2], Adelar Bracht [1,*], and Rosane Marina Peralta[1]

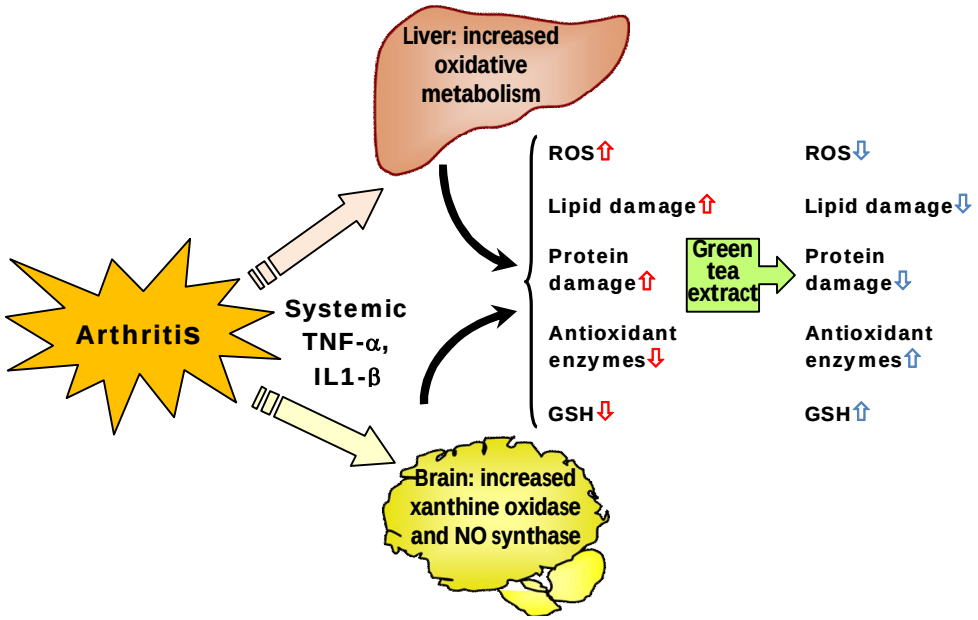
[1] Department of Biochemistry, State University of Maringá, 87020900 Maringá, Brazil

[2] Department of Pharmacology and Therapeutics, State University of Maringá, 87020900 Maringá, Brazil

* Corresponding author: adebracht@uol.com.br

Table of contents entry

Green tea improves the oxidative state of liver and brain of rats with adjuvant-induced arthritis and restores the antioxidant defenses.



Abstract

The purpose of the study was to evaluate the possible effects of the administration of a green tea extract on the oxidative state of the liver and brain of adjuvant-induced arthritic rats, a model for human rheumatoid arthritis. Daily doses of 250 mg/Kg (59.8 mg catechins per Kg) during 23 days were administered. This treatment produced significant diminutions in protein and lipid damage in liver, brain and plasma. It also diminished the tissue ROS contents and increased the antioxidant capacity of the plasma. The antioxidant defenses, which are diminished by arthritis, were improved by the green tea treatment, as revealed by the restoration of the GSH and protein thiol levels and by the strong tendency of normalizing the activities of the antioxidant enzymes. The activity of glucose 6-phosphate dehydrogenase, which is increased by arthritis in the liver, was also almost normalized by the treatment. In conclusion, it can be said that green tea consumption is possibly beneficial for the liver and brain of patients suffering from rheumatoid arthritis because it attenuates the pronounced oxidative stress that accompanies the disease and, thus, diminishes the injury to lipids and proteins in both liver and brain. There are also indications that, in the liver, the green tea can contribute to normalize the metabolic functions that are substantially modified by arthritis. For example, the green tea normalized the activity of glucose 6-phosphate dehydrogenase, a key enzyme of an important metabolic route (pentose monophosphate pathway). It is to be expected that the green tea treatment is equally able to normalize the activity of other enzymes (e.g., glucokinase and glucose 6-phosphatase), a hypothesis to be tested by future work.

Keywords: arthritis, liver, brain, green tea, antioxidants, catechins, redox status.

Introduction

Rheumatoid arthritis is a symmetric polyarticular arthritis that primarily affects the small diarthrodial joints of the hands and feet. Is a chronic inflammatory autoimmune mediated disorder characterized by cellular infiltration and proliferation of the synovial membrane, pannus formation, cartilage and bone erosion, leading to the progressive destruction of the joints through the interaction between infiltrating cells and mediators like cytokines, prostanoids and proteolytic enzymes. Rheumatoid arthritis occurs in 0.5–1.0% of the adult population worldwide.[1]

The pathophysiology of arthritis involves an intense hyperplasia of the articular cartilage with participation of T cells, B cells, macrophages, fibroblasts, and proinflammatory cytokines, particularly interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α).[2] Disease progression is also attributed to increases in reactive oxygen species (ROS) and oxidative stress in the lesion sites.[3] Because rheumatoid arthritis is a multisystem disease, in addition to the joints it also affects other organs such as liver, lung and vascular tissue.[4,5] The liver of rats with adjuvant-induced arthritis, for example, presents increased rates of oxygen uptake, inhibition of gluconeogenesis and stimulation of glycolysis, alterations in the urea cycle and modifications in calcium homeostasis.[6–10] A detailed investigation on the oxidative state of the liver of arthritic animals has been done more recently.[11] Substantial modifications have been detected, as for example higher levels of ROS, protein carbonyl groups and thiobarbituric acid reactive substances (TBARS) in several subcellular fractions (cytosol, mitochondria and peroxisomes). It was proposed that these modifications in the liver of arthritic rats are mainly consequence of the metabolic alterations caused by the disease especially the increased oxidative metabolism.[11] Similar observations were made in the brain of arthritic rats, although the causes in this tissue are apparently much less dependent on an increased oxidative metabolism than in the liver.[12]

Green tea has been shown to have several health beneficial effects, which have been attributed to its content in polyphenols, mainly catechins and catechin derivatives, including (-)-epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC) and epicatechin-3-gallate (ECG).[13] Green tea extracts or catechin-rich preparations have been proposed to be the useful in the prevention and/or

treatment of various disorders including cancer, cardiovascular disease, diabetes mellitus and hepatitis.[14–21] Extracts of green tea have been shown to inhibit the inflammatory responses and the development of arthritis in animal model studies.[17] The free radical scavenging action of the catechins as well as their ability to chelate transition metals such as iron and copper can be expected to prevent the formation of reactive oxygen species and inhibit lipid peroxidation in addition of increasing the expression of antioxidant enzymes.[13,22] Several studies have emphasized a possible beneficial action of green tea extracts or catechin enriched preparations on arthritis inflammatory states or senescence-induced changes in the brain antioxidant abilities.[23–26]

Although it is a generally accepted notion that the polyphenols of green tea should be effective on the oxidative stress that accompanies arthritis and other diseases, systematic studies on this question are lacking for most cells and tissues, except perhaps human chondrocytes and a few other models of oxidative stress.[17,27,28] This is especially true for the liver and brain, which have only recently been investigated systematically on this respect.[11,12] For this reason we decided to investigate the actions of a green tea extract on the modifications in the oxidative status caused by arthritis in these two tissues. The experimental system was the adjuvant-induced arthritic rat. The adjuvant-induced arthritis is an experimental immunopathology in rats that presents similarities to human rheumatoid arthritis: synovial hyperplasia, systemic inflammation, cachexia, and high levels of plasma pro-inflammatory cytokines and lesion sites.[29,30] The data obtained should further contribute to our current knowledge about the oxidative state of the liver and brain during arthritis and about the therapeutic and physiologic abilities of green tea.

Materials and Methods

Chemicals

Dinitrophenylhydrazine (DNPH), 2′7′-dichlorofluoresceindiacetate (DCFH-DA), oxidized dichloro-fluorescein (DCF), 1,1′,3,3′-tetraethoxypropane, horse-radish peroxidase (HRP), *o*-phthalaldehyde (OPT), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GRd), 2,2-azinobis 3-ethyl-benzothiazoline-6-sulfonate (ABTS), complete Freund's adjuvant (CFA), 5,5-dithiobis 2-nitrobenzoic acid (DTNB), catechins (EGCG, EC and C), caffeine and nitrate reductase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The commercial kit for albumin assay was purchased from Gold Analisa Diagnóstica Ltda (Belo Horizonte, Brazil). All other chemicals were of analytical grade.

Preparation of the *Camellia sinensis* aqueous extract and quantification of total phenolics

Leaves of *Camellia sinensis* var. *assamica*, originating from the Ribeira Valley (Brazil), were purchased in supermarkets (Maringá, PR, Brazil). The tea was prepared by infusing 1 g of the dried leaves per 100 ml distilled water at 80 °C for 8 min under constant stirring. The extract was then filtered though filter paper using a vacuum pump. The filtrates were lyophilized and stored in freezer until use. The concentration of the phenolic compounds in the extract was determined by the Folin-Ciocalteu method, using catechin as a standard.[31] The absorbance was measured at 725 nm. The results were expressed as µg catechin per mg of dry extract.

Determination of the polyphenol contents in green tea extract

The extract was analyzed by high performance liquid chromatography (HPLC). The analyses were carried out on a ShimadzuSPD-20A model. The column used was a 100-4 Superspher R C-18 (Macherey-Nagel, 125 x 2 mm, 4 mm). The mobile phase was water: acetonitrile:methanol:ethyl acetate: glacial acetic acid (89:6:1:3:1) with a flow rate of 0.7 ml/min.[32,33] The spectrophotometric detection was at 280 nm and the temperature 20±2 °C. Identification of the detected peaks was carried out by comparison of their retention times with those obtained by injecting standards in the same

conditions, as well as by spiking the green tea samples with stock standard solutions. Quantification of the identified compounds in the extract samples were calculated by means of the regression parameters obtained from calibration curves. The calibration curves were constructed by separation chromatographically standard solutions of the compounds. Linear relationships were obtained between the concentrations and areas under elution curves.

Animals and treatments

Male Holtzman rats were fed *ad libitum* with a standard laboratory diet (Nuvilab, Colombo, Brazil) and maintained on a regulated light dark cycle. For the induction of adjuvant arthritis, animals weighing 180-210 g were injected in the left hind paw with 0.1 ml of Freund's complete adjuvant (heat-inactivated *Mycobacterium tuberculosis*, derived from the human strain H37Rv), suspended in mineral oil at a concentration of 0.5% (w/v).[34] Rats of similar weights served as controls. All experiments of adjuvant arthritis induction were done in accordance with the world-wide accepted ethical guidelines for animal experimentation and previously approved by the Ethics Committee for Animal Experimentation of the University of Maringá (Protocol nº 067/2014-CEUA-UEM).

The animals were divided into 4 groups (6 rats each): (1) group I were healthy animals (controls); (2) group II were healthy animals treated with green tea extract (treated controls); (3) group III were the arthritic rats; (4) group IV comprised the arthritic rats treated with the green tea extract (arthritic treated). The treatment of the arthritic rats consisted in the administration of the green tea extract during 5 days prior to the induction of arthritis and during additional 18 days after initiating the induction. The daily doses were 250 mg extract per Kg body weight. The treated controls received the same doses during 23 days. Control animals, healthy or arthritic, received daily distilled water for 23 days.

Preparation of liver and brain homogenates

After 23 experimental days the 18 hours fasted rats were decapitated, the brain and the liver were immediately removed, freeze-clamped, and stored in liquid nitrogen. The tissues were then homogenized separately in a van Potter homogenizer with 10 volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and aliquots were separated for use as total homogenates. The remaining

homogenates were centrifuged at 11,000*g* during 15 min and the supernatants separated as the soluble fractions of the homogenates.

Blood collection and plasma preparation

After 23 experimental days the 18 hours fasted rats were were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}) and the blood was collected by puncturing the inferior vena cava after laparotomy and transferring it to tubes containing anticoagulant. The samples were centrifuged at 1,000*g* for 10 minutes to separate the plasma fraction. Albumin and total proteins were assayed by spectrophotometric techniques using commercial kits.

Oxidative injury parameters

The brain and the liver homogenate were assayed for lipid peroxidation by means of the TBARS assay (thiobarbituric acid reactive substances).[35] The amount of lipoperoxides was calculated from the standard curve prepared with 1,1',3,3'-tetraethoxypropane and the values were expressed as nmol (mg protein)$^{-1}$. The protein carbonyl groups contents were measured spectrophotometrically in the liver and brain homogenates and in the plasma using 2,4-dinitrophenylhydrazine (DNPH) (ε_{370} = 22 x 10^3 M^{-1} cm^{-1}) and the values were expressed as nmol (mg protein)$^{-1}$.[36]

Determination of the total antioxidant capacity (TAC)

The total antioxidant capacity of the plasma was determined colorimetrically with 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid (ABTS).[37] Plasma aliquots (50 µL) were added to 1.8 mL of 0.4 M acetate buffer (pH 5.8) plus 150 µL of a cationic ABTS solution (30 mM acetate buffer, pH 3.6, containing 10 mM ABTS and 4 mM H_2O_2). After 5 minutes of incubation in the dark, the absorbance at 734 nm was read against water. The compound 6-hydroxy-2,5,7,8-tetra-methyl-chloraman-2-carboxylic acid (TROLOX) was used as a standard and the results were expressed as µmol TROLOX equivalents per mg protein.

Protein reduced thiol groups measurement

The reduced protein thiol groups in the plasma were determined using the compound 5,5'-dithiobis 2-nitrobenzoic acid (DTNB).[38] Proteins in 100 µL of the plasma were precipitated with 1.0 mL 5% trichloroacetic acid + 5 mM EDTA.

After centrifugation at 2000*g* for 3 minutes, the precipitate was homogenized with a pellet homogenizer. The process of precipitation/homogenization was repeated twice and the final precipitate was suspended in 3.0 mL of 0.1 M TRIS buffer (pH 7.4) containing 5 mM EDTA and 0.5% sodium dodecyl sulfate. An aliquot of 400 µL of this solution was transferred to 1.6 mL of 0.1 M TRIS buffer (pH = 8.6) containing 5 mM EDTA with a further addition of 20 µL of 10 mM DTNB. After 10 minutes in the dark, the absorbance against blank was determined at 412 nm. The blank consisted in 2.0 mL 0.1 M TRIS buffer (pH = 8.6) plus 20 µL of 10 mM DTNB. The concentration of reduced thiols was calculated using a molar extinction coefficient of 1.36 x 10^4 M^{-1} cm^{-1} and expressed as nmol per mg protein.

ROS measurement

Reactive oxygen species were assessed in the homogenate supernatants. The total ROS content was quantified via the 2'-7'-dichlorofluorescein-diacetate (DCFH-DA) assay.[39] Acetate groups of DCFH-DA allow it to enter the organelles. These groups are removed by esterases producing the reduced DCFH within the organelle, which can be oxidized by peroxides to the fluorescent oxidized dichlorofluorescein (DCF). The formation of DCF was measured immediately after stopping the reaction on ice with a spectrofluorimeter RF-5301 (Shimadzu) in which the excitation and emission wavelengths were set at 504 and 529 nm, respectively. A standard curve with oxidized dichlorofluorescein (DCF) was used to express the results as nmol (mg of protein)$^{-1}$.

Glutathione assay

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in the total homogenate. The GSH and GSSG contents were measured spectrofluorimetrically (excitation 350 nm and emission 420 nm) by means of the *o*-phytalaldehyde assay.[40] Fluorescence was estimated as GSH. For the GSSG assay, the sample was previously incubated with 10 mM N-ethylmaleimide (NEM) and subsequently with a mixture containing 1 M NaOH and 0.4 µM phthalaldehyde to detect the fluorescence. The results were calculated using a standard curve prepared with GSH or GSSG and the values were expressed as µmol per gram brain or, alternatively, as nmol (mg protein)$^{-1}$.

Nitrite plus nitrate contents

The nitrite plus nitrate contents were measured in the total liver and brain homogenates. Nitrate was first converted into nitrite by adding the enzyme nitrate reductase and the total nitrite was quantified by the Griess method.[41] The results were expressed as nmol (mg of protein)$^{-1}$.

Enzyme assays

Antioxidant enzymatic activities were assessed in the homogenate supernatant. The catalase (CAT) activity was estimated by measuring changes in absorbance at 240 nm using H_2O_2 as substrate and expressed as µmol min^{-1} (mg protein)$^{-1}$.[42] The glutathione redutase (GR) activity was estimated by measuring changes in absorbance at 340 nm using NADPH and GSSG as substrates and expressed as nmol min^{-1} (mg protein)$^{-1}$.[42] The superoxide dismutase (SOD) activity was estimated by its capacity of inhibiting the pyrogallol autoxidation in alkaline medium. The latter was measured at 420 nm.[43] One SOD unit was considered the quantity of enzyme that was able to promote 50% inhibition and the results were expressed as Units (mg protein)$^{-1}$. The glutathione peroxidase (GPx) activity was estimated by measuring changes in absorbance at 340 nm due to NADPH consumption in the presence of H_2O_2, GSH and glutathione redutase and expressed as nmol min^{-1} (mg protein)$^{-1}$.[44] The activity of glucose 6-phosphate dehydrogenase was measured as the reduction rate of $NADP^+$ in the presence of glucose 6-phosphate.[42] The increase in absorbance due to NADPH production was monitored during three minutes. The initial rates were obtained by extrapolation to zero time and the activity computed as nmol min^{-1} mg protein^{-1} using the molar extinction coefficient of NADPH (6.22×10^3 M^{-1} cm^{-1}).

Statistical analysis

The error parameters presented in graphs and tables are standard errors of the **m**eans. Statistical analysis was done by means of the GraphPad Prism Software (version 5.0). Evaluation of the statistical significance was done by means the variance analysis (ANOVA) followed by post hoc Student-Newman-Keuls testing. The 5% level ($p<0.05$) was adopted as the significance criterion.

Results

Catechin content of the green tea extract

The catechin contents of green tea extracts and infusions has been measured in numerous studies.[18,21,26,45] Similar measurements were done in the present work to characterize the specific sample that was used and also to provide a basis for comparisons with other studies. The results are shown in Table 1. Epigallocatechingallate (EGCG) was the most abundant, accounting for nearly 50% of the total catechin content. The total catechin content revealed by the HPLC analysis (239.33 µg/mg; Table 1), was close to the total phenolic content found by using the Folin-Ciocalteu assay which was equal to 297.71±3.43 µg/mg (n=3).

Effects of the green-tea extracts on oxidative stress indicators

The results of the measurements that were done in the liver of healthy and arthritic rats are shown in the four panels of Figure 1. Treatment of healthy rats with the green tea extract did not produce any modification in the four parameters that were measured in the liver, protein carbonyls (panel A), reactive oxygen species (ROS; panel B), lipid peroxidation (TBARS; panel C) and the nitrate + nitrite content (panel D). Arthritis increased protein carbonyls (74%), ROS (72%) and TBARS (59.5%). The small increment in the nitrate + nitrite contents (13%) lacks statistical significance. These results confirm previous reports.[11,12] Treatment of arthritic rats with the green tea extract diminished the three parameters that were increased by the disease. The arthritis-induced increases in the protein carbonyl levels, ROS and TBARS were diminished by 56.9%, 44% and 59%, respectively. These numbers were calculated as 100 × (1 – percent increase in treated arthritic rats/percent increase in non-treated arthritic rats).

Figure 2 shows the corresponding measurements that were done in the brain. In this tissue the treatment of healthy rats was also without effect on the four parameters that were measured. Arthritis produced changes that were qualitatively similar to those found for the liver but quantitatively somewhat less pronounced: a 41% increase in the protein carbonyls (panel A), 39% in the ROS levels (panel B), 43% in the TBARS levels (panel C) and no significant change in the nitrate + nitrite content (panel D). Treatment of the arthritic rats with the

green tea extract also diminished these increases by 53.6%, 56.4% and 46.0 for the protein carbonyl levels, ROS and TBARS, respectively. The nitrate and nitrite levels of the brain showed no significant changes as revealed by panel D in Figure 2.

The plasma parameters measured in the present work were protein carbonyls, the total antioxidant capacity, protein thiols and the albumin concentration. The results are shown in Figure 3. Treatment of healthy rats with the green tea extract caused a small diminution in the plasma protein carbonyls (17.9%), as shown in Figure 3A. The other parameters were not changed by the green tea extract treatment of healthy rats. Arthritis, on the other hand, modified substantially all variables assayed in the present study. The protein carbonyl levels were increased by 34.3%. The total antioxidant capacity (TAC), the protein thiols and the albumin concentration were decreased by 26, 37.5, and 33.7%, respectively. All these parameters were improved by the green tea extract administration. The increase in the protein carbonyl content (Figure 3A) was diminished by 781%. The drops in the TAC, protein thiols and albumin concentration were reduced to 24, 66.6 and 56.6%, respectively, of those found in non-treated arthritic rats. These numbers were calculated as 100 × (percent decrease in treated arthritic rats/percent decrease in non-treated arthritic rats). In spite of the drop in the plasma albumin concentration in arthritic rats, the total protein concentration was increased by 15.6% (from 52.5±1.0 to 60.7±0.5 mg/mL; $p < 0.05$). In green tea-treated rats this increase was significantly reduced to 8.8% relative to the healthy rat (57.1±0.7 mg/mL; $p < 0.05$).

GSH and GSSG tissue contents

The concentrations of both reduced and oxidized forms of glutathione (GSH and GSSG) were measured in both liver and brain. The results, in all cases expressed as nm per mg protein, are listed in Table 2. Administration of the green tea extract to healthy rats caused increases in the GSH contents of both liver and brain (19.7 and 27.3%, respectively). In the brain the GSSG content of healthy rats was also increased (21.7%). The GSH/GSSG ratios in brain and liver of healthy rats, however, were not significantly modified by the green tea extract administration. Arthritis, on the other hand, clearly decreased the GSH content, by 34.5% in the liver and 31.1% in the brain. This phenomenon was coupled in the liver to a substantial increase in the GSSG content (+50%), but a decreasing

tendency was observed in the brain. In consequence, the GSH/GSSG ratio was much more decreased in the liver than in the brain. For both liver and brain these results confirm previous observations of our laboratory.[11,12] Administration of the green tea extract to arthritic rats was notable in that it virtually preserved the GSH content and the GSH/GSSG ratio in both tissues.

Enzyme activities

Five enzymatic activities related to the oxidative state of both tissues, liver and brain, were measured. The results are summarized in Table 3. The administration of the green tea extract to healthy rats did not produce statistically significant changes in any of the enzymatic activities measured in the present work. Arthritis, however, diminished the activities of catalase (liver 74.3%, brain 21.7%), glutathione reductase (liver 33.1%, brain 18.9%), superoxide dismutase (SOD; liver 31.1%, brain 27.0%) and glutathione peroxidase (liver 32.3%, brain 27.0%). The glucose 6-phosphate dehydrogenase, however, was increased in the liver (82.0%) and decreased in the brain (19.6%). Treatment of arthritic rats had a positive influence on practically all the enzymatic activities measured in the present work in both tissues. In the liver the diminishing influence of arthritis on catalase, glutathione reductase, superoxide dismutase and glutathione peroxidase was attenuated by the green tea treatment. The attenuation amounted to 34.3, 54.7, 45.3, and 65.0%, respectively. In the liver, also, the green tea treatment restrained the increase in the glucose 6-phosphate dehydrogenase activity, which was reduced to only 32.3%. In the brain, upon green tea treatment, the diminishing influence of arthritis on catalase, glutathione reductase, superoxide dismutase, glutathione peroxidase and glucose 6-phosphate dehydrogenase was attenuated by 80.4, 100.0, 82.4, 60.7% and 65.9%, respectively. Comparison of the effects of the green tea extract in the liver and brain of arthritic rats reveals that in the latter it was considerably more effective in relative terms.

Discussion

The importance of green tea as a functional food can hardly be over-emphasized as it is being continuously corroborated by numerous studies with several species and experimental systems. With specific reference to rheumatoid arthritis it has been shown in animal models that the development of the disease was ameliorated by the prophylactic administration of green tea polyphenols in the drinking water.[23,24] The present study adds more evidence to the health beneficial effects of green tea, in this case, as a formulation capable of attenuating the pronounced oxidative stress caused by arthritis in the liver and brain. Administration of the green tea extract proved to be effective in improving the oxidative state in these two tissues and it was also active on four blood parameters. Since this action requires the participation of antioxidant compounds, the extract used in the present study was characterized in terms of its contents in catechins and total phenolics, which are believed to be the most important antioxidant agents of green tea.[13,46] The extract doses that were administered daily, 250 mg/Kg, correspond to relatively low doses of both total phenolics and catechins, more precisely, 74.4 and 59.8 mg/Kg. These values are considerably lower, for example, than the minimal dose, 125 mg/kg, used in another study in which the green tea extract proved to be efficient in mitigating the senescence-induced changes in the brain antioxidant capacities of mice.[26]

The green tea extract administration diminished the arthritis-induced damage to both proteins and lipids in the liver as well as in the brain. In addition, it diminished the levels of reactive oxygen species also in both tissues. It was without statistically significant effects on the indicators (nitrate + nitrite) of the NO levels in the brain and liver homogenates. It should be noted, however, that modifications in the levels of nitrate + nitrite in consequence of arthritis were found only in subcellular fractions (mitochondria and peroxisomes), which comprise a small fraction of the total tissue.[11,12] One should not exclude, thus, that green tea might also be effective on these modifications. The results of this work are similar in many aspects to the observations that green tea exhibits protective effects against the senescence mediated redox imbalance in mice.[26] They are also consistent with the observations that treatment of hemodialysis patients reduces mononuclear cell expression of the oxidative stress related protein $p22^{phox}$ and the plasma fibrinogen levels.[25] The increased oxidative

damage in the liver of adjuvant-induced arthritic rats has been shown to be consistent with a more intense oxidative metabolism, which in turn, is expected to generate a more oxidizing environment and a more intense production of ROS.[11] In fact, the liver of arthritic rats presents higher activity of glucokinase and a diminished activity of glucose 6-phosphatase, a combination that leads to higher levels of glucose 6-phosphate and also to higher rates of glycolysis.[8,47] The pentose-monophosphate shunt should also be increased, as can be expected from the higher levels of glucose 6-phosphate combined with the increased activity of glucose-6-phosphate dehydrogenase (Table 3).[11,47] Perfused livers from arthritic rats are generally characterized by higher rates of oxygen uptake under several conditions.[6] Although this can be brought about by an increased flux of reducing equivalents from the cytosol in the case of increased glycolysis, there is equally evidence for an increased activity of the mitochondrial respiratory chain, an observation that is consistent with the reported increased cytochrome c oxidase activities.[6,11] In addition to the more intense oxidative metabolism in the liver of arthritic rats, the latter also present a deficient antioxidant defense, as indeed corroborated by the results of the present work which revealed reduced levels of antioxidant enzymes and a diminished GSH content. An impaired antioxidant defense in arthritic rats is also evident in the brain and in the plasma, as revealed by the smaller levels of protein thiols and the reduced total antioxidant capacity. Taking into account the two concomitant causes for the pronounced oxidative stress in arthritic rats, the main question that can be formulated is one about the mode of action of the green tea extract, which could occur (factor 1) by improving the antioxidant defenses, (factor 2) by damping the events which, at least in the liver, generate a more intense oxidative metabolism, or by a combination of both.[11] The data that are available so far do not allow to conclude with certainty about factor 2, i.e., there is still little information about the action of green tea on the oxidative metabolism. An indication that it might be acting on oxidative pathways is the diminution of the glucose 6-phosphate dehydrogenase activity in the liver of arthritic rats that was observed in the present work and which should mean a diminution of the flux through the pentose–monophosphate shunt. In this respect it should be mentioned that higher activities of glucose 6-phosphate dehydrogenase are generated by agents that promote oxidative stress in the rat liver, such as hydrogen peroxide and t-butylhydroperoxide.[48] Besides the activity of glucose 6-

phosphate dehydrogenase activity, thus, only specific measurements of the influence of green tea can provide further insights into the question if this preparation is able to diminish the excess respiratory activity of the liver or other cells during arthritis.

Concerning factor 1, the improvement of the antioxidant defenses, the action of green tea seems to be quite evident. The ingestion of the green tea extract significantly improved the levels of antioxidant enzymes in both brain and liver. It also improved the tissue concentration of reduced glutathione and the content of reduced protein thiols in the plasma. The combination of increased glutathione peroxidase activity with increased GSH levels in both tissues, brain and liver, are particularly important, because this enzyme actually degrades GSH in parallel with its activity in removing active oxygen species or their precursors (e.g., H_2O_2).[11,12] Higher levels of GSH in parallel with a higher glutathione peroxidase activity in arthritic rats, thus, also means a restored increased capacity of regenerating GSH from GSSG. This is partly justified by the almost restored activities of glutathione reductase in green tea-treated arthritic rats, but it could also mean a more reduced state of the $NADP^+$/NADPH and NAD^+/NADH couples induced by the various green tea components.[11,12]

The green tea administration to arthritic rats had also a positive influence on the plasma albumin concentration. Hypoalbuminemia is a common phenomenon in arthritic patients.[49] The albumin concentration does not correlate with the indices of the disease activity. The lower levels of plasma albumin in arthritic patients is much more the result of an increased relative catabolic rate. The effects of the green tea extract observed in the present study could thus be the result of a partial inhibition of the catabolic degradation of albumin.

Conclusions

In conclusion, it can be said that green tea consumption is possibly beneficial for the liver and brain of patients suffering from rheumatoid arthritis because it attenuates the pronounced oxidative stress that accompanies the disease and, thus, diminishes the injury to lipids and proteins in both liver and brain. There are also indications that, in the liver, the green tea can contribute to normalize the metabolic functions that are substantially modified by arthritis to the point that it is usual to classify these modifications as a cachectic state.[6–10] For example, green tea normalized the activity of glucose 6-phosphate dehydrogenase in the liver, a key enzyme of an important metabolic route (pentose monophosphate pathway). Based on this observation, one can expect that the green tea treatment should be equally able to normalize the activity of other enzymes (e.g., glucokinase and glucose 6-phosphatase), a hypothesis to be tested by future work.[8,47]

Acknowledgments

This work was financially supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação do Aperfeiçoamento de Pessoal do Ensino Superior (CAPES).

Conflicts of Interest

The authors declare no conflict of interest.

References

1. G.S. Firestein, Evolving concepts of rheumatoid arthritis. *Nature,* 2003, **423,** 356–361.
2. E. H. S. Choy, G. S. Panayi, Cytokine pathways and joint inflammation in rheumatoid arthritis. *New Engl. J. Med.,* 2001, **344**, 907–916.
3. A. Seven, S. Güzel, M. Aslan, V. Hamuryudan, Lipid, protein, DNA oxidation and antioxidant status in rheumatoid arthritis. *Clin. Biochem.,* 2008, **41**, 538–543.
4. O. Sangha, Effect size in clinical studies of patients with rheumatoid arthritis. EULAR guidelines and OMERACT core-sets. *Z. Rheumatol.,* 2000, **59**, 45–49.
5. N. Sattar, D.W. McCarey, H. Capell, I. McInnes, Explaining how “high-grade” systemic inflammation accelerates vascular risk in rheumatoid arthritis. *Circulation,* 2003, **108**, 2957–2963.
6. S.M. Caparroz-Assef, C.A. Bersani-Amado, E.A. Nascimento, A.M. Kelmer-Bracht, E.L. Ishii-Iwamoto, Effects of the nonsteroidal anti-inflammatory drug nimesulide on energy metabolism in livers from adjuvant-induced arthritic rats. *Res. Commun. Mol. Pathol. Pharmacol.,* 1998, **99***,* 93–116.
7. Z. Fedatto-Jr, E.L. Ishiii-Iwamoto, C.C. Amado, G. Vicentini, A. D’Urso-Panerari, A. Bracht, A.M. Kelmer-Bracht, Gluconeogenesis in the liver of arthritic rats. *Cell Biochem. Funct.,* 1999, **17**, 271–278.
8. Z. Fedatto-Jr, E.L. Ishii-Iwamoto, C. Bersani-Amado, E.R.M. Maciel, A. Bracht, A.M. Kelmer-Bracht, Glucose phosphorylation capacity and glycolysis in the liver of arthritic rats. *Inflamm. Res.,* 2000, **49**, 128–132.
9. P. Yassuda-Filho, A. Bracht, E.L. Ishii-Iwamoto, S.H. Lousano, L. Bracht, A.M. Kelmer-Bracht, The urea cycle in the liver of arthritic rats. *Mol. Cell. Biochemistry,* 2003, **243**, 97–106.
10. K.S. Utsunomiya, L.G. Scaliante, A. Bracht, E.L. Ishii-Iwamoto, Transport and distribution of $^{45}Ca^{2+}$ in the perfused rat liver and the influence of adjuvant-induced arthritis. *BBA-Mol. Basis Dis.,* 2013, **1832**, 249–262.
11. J.F. Comar, A.B. Sá-Nakanishi, A.J. Oliveira, M.M.N. Wendt, C.A.B. Amado, E.L. IshiiIwamoto, R.M. Peralta, A. Bracht, Oxidative state of the liver of rats with adjuvant-induced arthritis. *Free Rad. Biol. Med.,* 2013, **58,** 144–153.
12. M.M.N. Wendt, A.B. Sá-Nakanishi, C.V.C. Ghizoni, C.A.B. Amado, R.M. Peralta, A. Bracht, J.F. Comar, Oxidative state and oxidative metabolism in

the brain of rats with adjuvant-induced arthritis. *Exper. Mol. Pathol.,* 2015, **98**, 549–557.

13. S.P.J. Namal Senanayake, Green tea extract: chemistry, antioxidant properties and food applications – A review. *J. Funct. Foods,* 2013, **5**, 1529–1541.
14. A. Rietveld, S. Wiseman, Antioxidant effects of tea: evidence from human clinical trials. *J. Nutr.,* 2003, **133**, 3285S–3292S.
15. J.K. Willcox, S.L. Ash, G.L. Catignani, Antioxidants and prevention of chronic disease. *Crit. Rev. Food Sci. Nutr.,* 2004, **44**, 275–295.
16. K. Boehm, F. Borrelli, E. Ernst, G. Habacher, S. Kang Hung, S. Milazzo, M. Horneber, Green tea (Camellia sinensis) for the prevention of cancer. *Cochrane DB Syst. Rev.,* 2009, **3**, 1–58.
17. R. Singh, N. Akhtar, T.M. Haqqui, Green tea polyphenol epigallocatechin-3-gallate: inflammation and arthritis. *Life Sci.,* 2010, **86**, 907–918.
18. Y. Zhong, Y.-S. Chiou, M.-H. Pan, C.-T. Ho, F. Shahidi, Protective effects of epigallo-catechingallate (EGCG) derivatives on azoxymethane-induced colonic carcinogenesis in mice. *J. Funct. Foods,* 2012, **4**, 323–330.
19. W. Tang, S. Li, Y. Liu, M.-T. Huang, C.-T. Ho, Anti-diabetic activity of chemically profiled green tea and black tea extracts in a type 2 diabetes mice model via different mechanisms. *J. Funct. Foods,* 2013, **5**, 1784–1793.
20. C.-F. Tsai, Y.-W. Hsu, H.-C. Ting, C.-F. Huang, C.-C. Yen, The in vivo antioxidant and antifibrotic properties of green tea (Camellia sinensis, Theaceae). *Food Chem.,* 2013, **136,** 1337–1344.
21. Y. Fukuzawa, M.P. Kapoor, K. Yamasaki, T. Okubo, Y. Hotta, L.J. Juneja, Effects of green tea catechins on nonalcoholic steatohepatitis (NASH) patients. *J. Funct. Foods,* 2014, **9**, 48–59.
22. Q. Meng, C.N. Velalar, R. Ruan. Effects of epigallocatechin-3-gallate on mitochondrial integrity and antioxidative enzyme activity in the aging process of human fibroblast. *Free Rad. Biol. Med.,* 2001, **44**, 1032–1041.
23. T.M. Haqqi, D.D. Anthony, S. Gupta, N. Ahmad, M.S. Lee, G.K. Kumar, H. Mukhtar, Prevention of collagen-induced arthritis in mice by a polyphenolic fraction from green tea. *Proc. Natl. Acad. Sci. U.S.A.,* 1999, **96**, 4524–4529.
24. A. Morinobu, W. Biao, S. Tanaka, M. Horiuchi, L. Jun, G. Tsuji, Y. Sakai, M. Kurosaka, S. Kumagai, (–)-Epigallocatechin-3-gallate suppresses osteoclast

differentiation and ameliorates experimental arthritis in mice. *Arthr. Rheum.,* 2012, **58**, 2012–2018.

25. U. Vertolli, P.A. Davis, L.D. Maso, G. Maiolino, A. Naso, M. Plebani, L.A. Calò, Daily green tea extract supplementation reduces prothrombotic and inflammatory states in dialysis patients. *J. Funct. Foods,* 2013, **5**, 1366–1371.
26. Y.W. Hsu, C.F. Tsaib, H.C. Tingb, W.K. Chenc, C.C. Yena, Green tea supplementation in mice mitigates senescence-induced changes in brain antioxidant abilities. *J. Funct. Foods,* 2014, **7**, 471–478.
27. D.U. Song, Y.D. Jung, K.O. Chay, M.A. Chung, K.H. Lee, S.Y. Yang, B.A. Shin, B.W. Ahn, Effect of drinking green tea on age-associated accumulation of Maillard-type fluorescence and carbonyl groups in rat aortic and skin collagen. *Arch. Biochem. Biophys.,* 2002, **397**, 424–429.
28. G. Nie, C. Jin, Y. Cao, S. Shen, B. Zhao, Distinct effects of tea catechins on 6-hydroxydopamine-induced apaptosis in PC12 cells. *Arch. Biochem. Biophys.,* 2002, 397, 84–90.
29. A. Bendele, J. Mccomb, T. Gould, T. Mcabee, G. Sennello, E. Chlipala, M. Guy, Animal models of arthritis: relevance to human disease. *Toxicol. Pathol.,* 1999, **27**, 134–142.
30. Z. Szekanecz, M.M. Halloran, M.V. Volin, J.M. Woods, R.M. Strieter, G.K. Haines, S.L. Kunkel, M.D. Burdick, A.E. Koch, Temporal expression of inflammatory cytokines and chemokines in rat adjuvant-induced arthritis. *Arthr. Rheum.,* 2000, **43**, 1266–1277.
31. V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Amer. J. Enol. Vitic.,* 1965, **16**, 144–158.
32. S.T. Saito, A. Welzel, E.S. Suyenaga, F. Bueno, A method for fast determination of epigallocatechin gallate (EGCG), epicatechin (EC), catechin (C) and caffeine (CAF) in green tea using HPLC. *Food Sci. Technol. (Campinas),* 2006, **26**, 394–400.
33. S.T. Saito, P.E. Fröehlich, G. Gosmann, A.M. Bergold, Full validation of a simple method for determination of catechins and caffeine in brazilian green tea (*Camellia sinensis* var. *assamica*) using HPLC. *Chromatographia,* 2007, **65**, 607–610.

34. C.M. Pearson, F.D. Wood, Studies of arthritis and other lesions induced in rats by the injection of mycobacterial adjuvant. *Am. J. Pathol. (Philadelphia),* 1963, **42**, 93–95.
35. J.A. Buege, S.D. Aust, Microsomal lipid peroxidation. *Meth. Enzymol.,* 1978, **52**, 302–310.
36. R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, S. Shaltiel, E.R. Stadtman, Determination of carbonyl content in oxidatively modified proteins. *Meth. Enzymol.,* 1990, **186***,* 464–478.
37. O. Erel, A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.,* 2004*,* **37**, 277–285.
38. J. Sedlak, R.H. Lindsay, Estimation of total, protein-bound, and non-protein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.,* 1968, **25**, 192–205.
39. I.R. Siqueira, C. Fochesatto, I.L. da Silva Torres, C. Dalmaz, C. Alexandre Netto, Aging affects oxidative state in hippocampus, hypothalamus and adrenal glands of Wistar rats. *Life Sci.,* 2005*,* **78**, 271–278.
40. P.J. Hissin, R. Hilf, A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.,* 1976, **74**, 214–226.
41. N.S. Bryan, M.B. Grisham, Methods to detect nitric oxide and its metabolites in biological samples. *Free Rad. Biol. Med.,* 2007, **43**, 645–657.
42. H.U. Bergmeyer, *Methods of Enzymatic Analysis*, Verlag Chemie-Academic Press: Weinheim, London, 1974.
43. S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.,* 1974, **47**, 469–474.
44. A.L. Tappel, Glutathione peroxidase and hydroperoxides. *Meth. Enzymol.,* 1978, **52**, 506–513.
45. H. Wang, G.J. Provan, K. Helliwell, HPLC determination of catechins in tea leaves and tea extracts using relative response factors. *Food Chem.,* 2003*,* **81***,* 307–312.
46. J.V. Higdon, B. Frei, Tea catechins and polyphenols: Health effects, metabolism and antioxidant functions. *Crit. Rev. Food Sci. Nutr.,* 2003*,* **43***,* 89–143.

47. A.M. Kelmer-Bracht, C.P.B. Santos, E.L. Ishii-Iwamoto, A.C. Broetto-Biazon, A. Bracht, Kinetic properties of the glucose 6-phosphatase the liver from arthritic rats. *BBA-Mol. Basis Dis.,* 2003, **1638**, 50–56.

48. M. Cascales, P. Martín-Sanz, D.G. Craciunescu, I. Mayo, A. Aguiliar, E. Robles-Chillide, C. Cascales, Alterations in the hepatic peroxidation mechanisms in thioacetamide-induced tumors in rats. *Carcinogenesis,* 1991, **12**, 233–240.

49. F.C. Ballantyne, A. Fleck, W.C. Dick, Albumin metabolism in rheumatoid arthritis. *Ann. Rheum. Dis.,* 1971, **30**, 265-270.

Table 1 The catechin contents of the green-tea extract administered to the rats in the present study. Determination was done by means o HPLC. The results are means ± mean standard errors of 3 determinations.

Compound	Content (µg/mg)	Percentage of total catechins
Epigallocatechin (EGC)	30.63±1.30	12.80
Epichatechin (EC)	17.95±0.80	7.50
Catechin (C)	6.22±0.09	2.60
Epigallocatechingallate (EGCG)	120.33±2.15	50.28
Galocatechingallate (GCG)	15.99±0.37	6.68
Epicatechingallate (ECG)	41.50±1.00	17.34
Catechingallate (CG)	6.7±0.13	2.80
Total catechins	239.33±5.84	100

Table 2 Effects of arthritis and green tea extract treatment on the contents of GSH and GSSG in liver and brain. Control and arthritic rats were treated with the aqueous extract of green tea (250 mg/kg) according to the experimental protocol described in the Materials and Methods section. The data represent the mean ± standard error of the mean from 5-6 animals. Values with the same superscript letters in the same line differ statistically from each other ($p < 0.05$).

Parameters	Tissue	Control	Treated control	Arthritic	Treated arthritic
GSH	Liver	$16.53 \pm 0.71^{a,b}$	$19.79 \pm 0.90^{b,d,e}$	$10.82 \pm 0.66^{a,c,d}$	$15.43 \pm 0.47^{c,e}$
(nmol· mg^{-1})	Brain	$8.88 \pm 0.61^{a,b}$	$11.31 \pm 0.53^{b,d,e}$	$6.06 \pm 0.47^{a,c,d}$	$8.51 \pm 0.82^{c,e}$
GSSG	Liver	1.4 ± 0.06^{a}	1.49 ± 0.08^{c}	$2.10 \pm 0.03^{a,b,c}$	1.26 ± 0.06^{b}
(nmol· mg^{-1})	Brain	0.46 ± 0.02^{a}	$0.56 \pm 0.03^{a,b,c}$	0.38 ± 0.01^{b}	0.40 ± 0.01^{c}
GSH + 2 × GSSG	Liver	19.40 ± 0.69^{a}	22.78 ± 1.00^{c}	$13.22 \pm 2.40^{a,b,c}$	17.95 ± 0.50^{b}
(nmol GSH units· mg^{-1})	Brain	$9.80 \pm 0.65^{a,b}$	$12.44 \pm 0.59^{b,d,e}$	$6.84 \pm 0.45^{a,c,d}$	$9.32 \pm 0.91^{c,e}$
GSH / GSSG	Liver	11.40 ± 0.89^{a}	13.23 ± 0.80^{c}	$5.40 \pm 0.28^{a,b,c}$	11.54 ± 0.29^{b}
	Brain	19.90 ± 0.73^{a}	20.53 ± 1.84^{c}	$14.32 \pm 1.12^{a,b,c}$	21.11 ± 2.08^{b}

Table 3 Effects of arthritis and green tea extract treatment on the activity of antioxidant enzymes in liver and brain. Control and arthritic rats were treated with the aqueous extract of green tea (250 mg/ kg) according to the experimental protocol described in the Materials and Methods section. The data represent the mean ± standard error of the mean from 5-6 animals. Values with the same superscript letters in the same line differ statistically from each other ($p < 0.05$).

Parameters	Tissue	Control	Treated control	Arthritic	Treated arthritic
Catalase	Liver	$877.0 \pm 33.3^{a,c}$	$892.6 \pm 39.78^{d,e}$	$224.7 \pm 17.6^{a,b,d}$	$449.0 \pm 38.3^{b,c,e}$
(µmol min^{-1} mg^{-1}	Brain	14.56 ± 0.58^{a}	$16.28 \pm 1.09^{c,d}$	$11.40 \pm 0.20^{a,b,c}$	$13.94 \pm 0.28^{b,d}$
GR ***(nmol min^{-1} mg^{-1})***	Liver	35.62 ± 2.73^{a}	31.96 ± 0.83^{c}	$23.82 \pm 1.34^{a,b,c}$	30.28 ± 1.17^{b}
	Brain	16.98 ± 0.46	19.20 ± 0.82^{b}	$13.76 \pm 1.04^{a,b}$	18.28 ± 1.52^{a}
SOD ***(U mg^{-1})***	Liver	2.4 ± 0.14^{a}	2.24 ± 0.02^{c}	$1.66 \pm 0.05^{a,b,c}$	2.00 ± 0.17^{b}
	Brain	1.48 ± 0.09^{a}	1.65 ± 0.10^{c}	$1.08 \pm 0.10^{a,\ b,c}$	1.41 ± 0.02^{b}
GPx	Liver	206.6 ± 4.6^{a}	224.0 ± 8.0^{c}	$139.8 \pm 21.3^{a,b,c}$	183.2 ± 9.4^{b}
(nmol min^{-1} mg^{-1})	Brain	26.44 ± 1.13^{a}	27.72 ± 1.69^{c}	$19.31 \pm 0.76^{a,b,c}$	23.63 ± 0.99^{b}
G6PDH	Liver	21.77 ± 1.65^{a}	26.84 ± 3.24^{c}	$39.62 \pm 4.77^{a,b,c}$	28.81 ± 0.38^{b}
(nmol $min^{-1}mg^{-1}$)	Brain	31.32 ± 1.70^{a}	30.05 ± 1.28^{c}	$25.17 \pm 1.17^{a,b,c}$	29.23 ± 1.11^{b}

Figure legends

Fig. 1 Treatment of normal and arthritic rats with the aqueous green tea extract: effects on hepatic oxidative stress indicators. Freeze-clamped rat livers were homogenized for the assays, as described in the Materials and Methods section. Values represent mean ± standard error of the mean from 6 animals for each experimental condition. Statistics: *$p \leq 0.05$ when compared to the control group; $^{\#}p \leq 0.05$ when compared to the arthritic group.

Fig. 2 Treatment of normal and arthritic rats with the aqueous green tea extract: effects on oxidative stress indicators in the brain. Freeze-clamped rat brains were homogenized for the assays, as described in the Materials and Methods section. Values represent mean ± standard error of the mean from 6 animals for each experimental condition. Statistics: *$p \leq 0.05$ when compared to the control group; $^{\#}p \leq 0.05$ when compared to the arthritic group.

Fig. 3 Treatment of normal and arthritic rats with the aqueous green tea extract: effects on plasmatic oxidative stress indicators and on the albumin concentration. The plasma samples were obtained and processed as described in Materials and Methods section. Values represent mean ± standard error of the mean of 6 animals for each experimental condition. Statistics: *$p \leq 0.05$ when compared to the control group; $^{\#}p \leq 0.05$ when compared to the arthritic group.

Figure 1

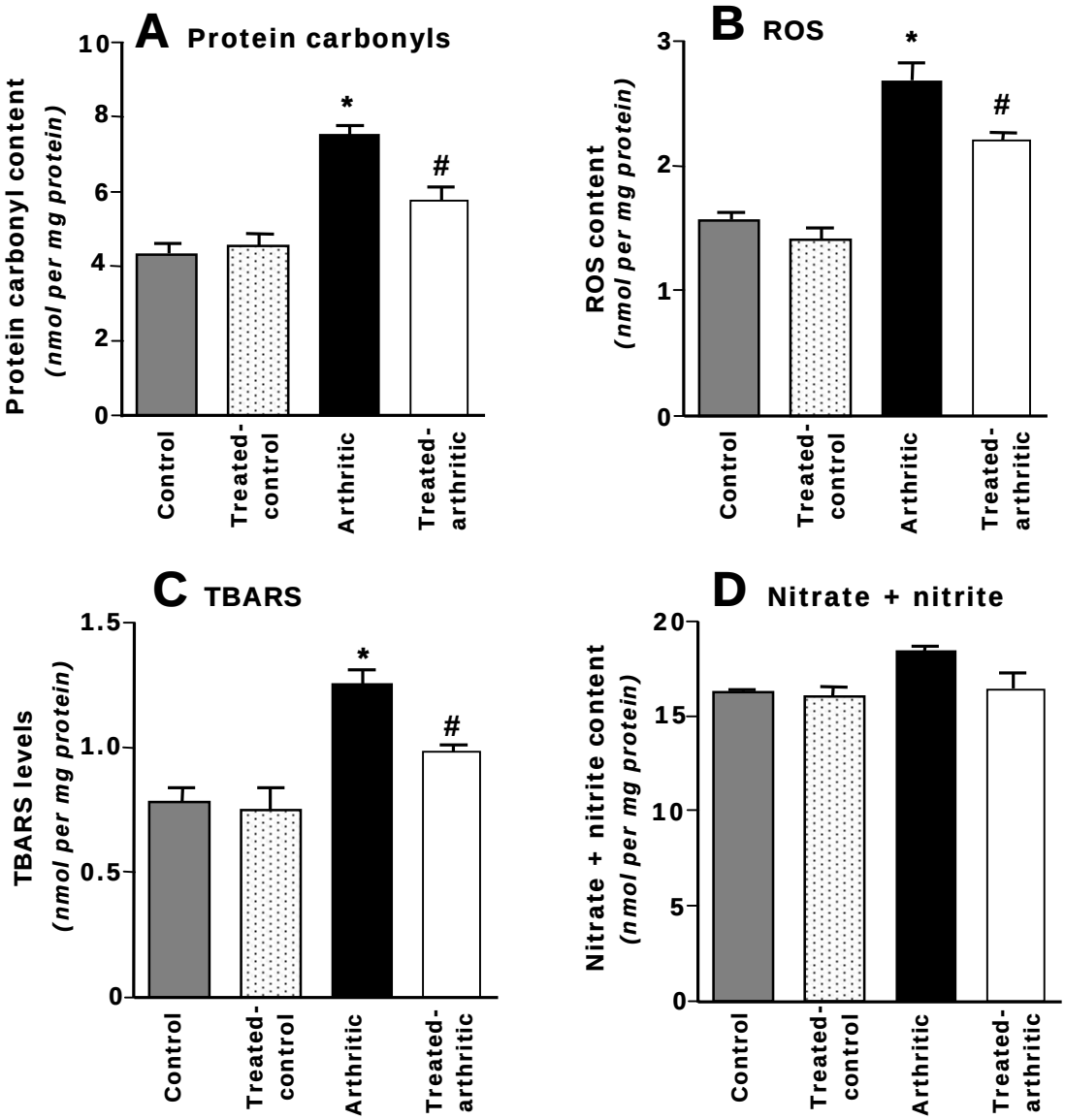


Figure 2

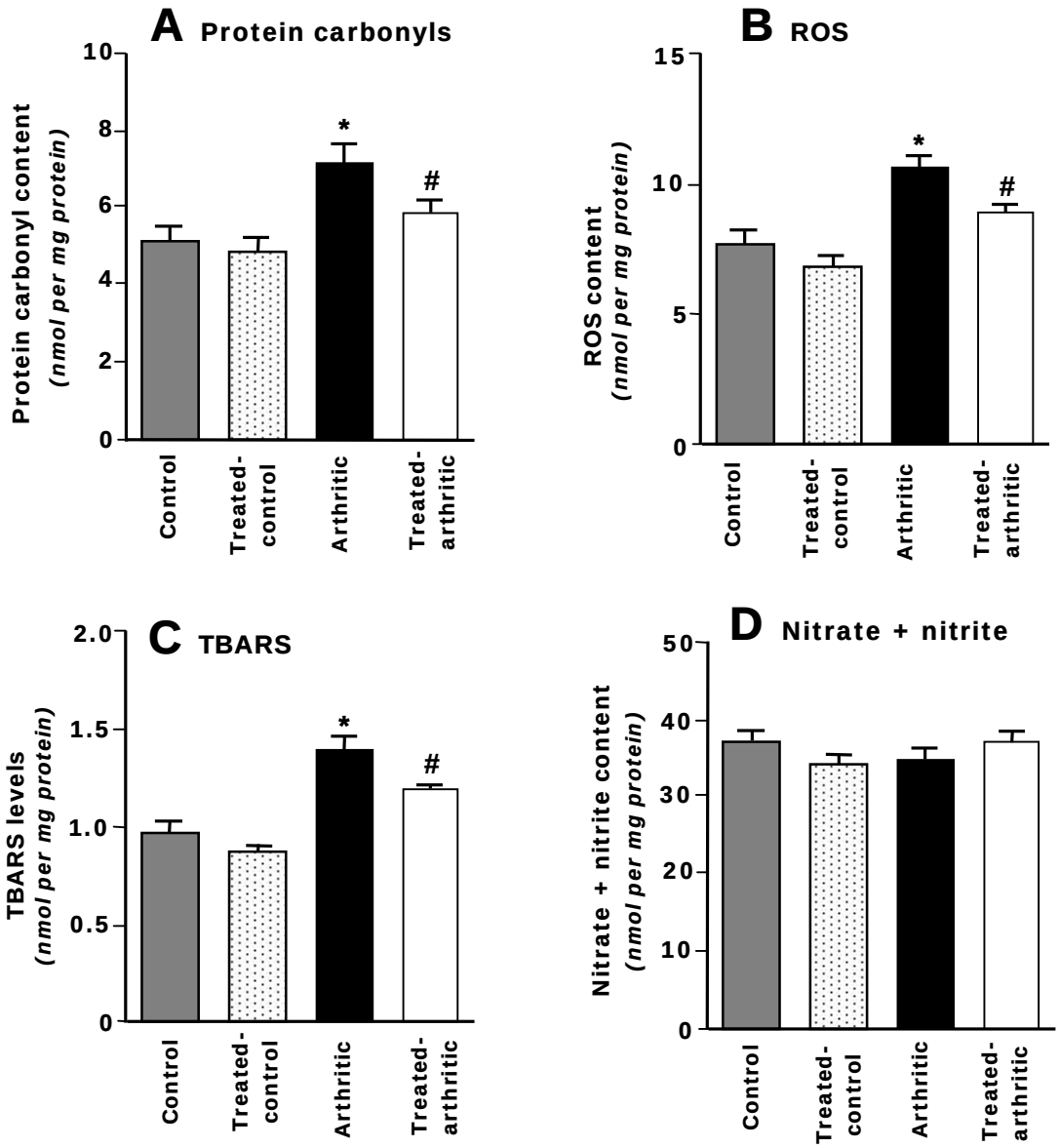


Figure 3