JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Increase of the Activity of Phase II Antioxidant Enzymes in Rats after a Single Dose of Coffee

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ABSTRACT: This study evaluated the acute effect of the administration of coffee brew in the activity of phase II antioxidant enzymes in the hepatic tissue of rats. A single dose of this beverage increased the activity of the enzymes SOD, CAT, and GPx; the maximum increase occurred 1 h after administration (19.1, 22.1, and 25.1%, respectively). These changes were statistically significant ($p < 0.05$), the response was shown to be dose-dependent ($p < 0.05$), and the return to basal levels took >4 h from the intervention, suggesting a long-term effect. The total antioxidant capacity of the hepatic tissue also exhibited a peak 1 h after the intervention (6.5%), but the increase was not statistically significant and the response was not dose-dependent due to the low exposure to coffee. These results indicate that coffee increases the activities of antioxidant enzymes, improving protection against oxidative stress.

KEYWORDS: coffee, oxidative stress, phase II enzymes, phenolic compounds, ORAC

INTRODUCTION

Oxidative stress is a condition characterized by an imbalance in the pro/antioxidant homeostasis, resulting from an excessive formation of reactive species such as free radicals or a low availability of antioxidants.[1−4]

In low/moderate concentrations, reactive species play important regulatory roles in the normal biological processes, activating different intracellular signaling and nuclear transcriptional factors, inducing mitogenic and inflammatory response, protecting against microbial infections, and regulating various enzymatic cascades.[3−6] However, several conditions such as lipid peroxidation, smoking, excessive consumption of alcohol, exposure to radiation or pollution, use of drugs, inflammations, and excessive exercise can increase their concentrations to dangerous levels.[7,8] Alzheimer's disease, atherosclerosis, cancer, cardiovascular diseases, asthma, emphysema, Parkinson's disease, schizophrenia, rheumatoid arthritis, lupus, cataracts, amyotrophic lateral sclerosis, and diabetes are some examples of human diseases already associated with oxidative stress.[2,6,7,9]

To restrain oxidative stress and its undesirable consequences, organisms have developed the capacity to synthesize endogenous antioxidants such as glutathione, bilirubin, dihydrolipoic acid, nicotinamide adenine dinucleotide phosphate (NADPH), thiol proteins, uric acid, melatonin, and ubiquinone,[2,8] as well as to use exogenous antioxidants acquired from the diet such as vitamins C and E, carotenoids, flavonoids, and several phenolic compounds.[2,5,7] In addition, the evolution endowed the organisms with the ability to transcript antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) that act to neutralize dangerous reactive species before they use their destructive capacity.[5−7]

It was already demonstrated that the administration of some phenolic acids such as gentisic, ferulic, gallic, and *p*-coumaric acids to rats over a period of 14 days largely increased the transcription and the activity of the enzymes SOD, CAT, and GPx.[10] A proposed mechanism suggests that the phenolic acids act as indirect inducers, activating kinases such as protein kinase C (PKC), phosphatidyl inositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) that phosphorylate the protein nuclear factor-E2-related factor (Nrf2), changing its conformation and dissociating it from the inhibitor cytoplasmic complex Kelch-like ECH-associated protein 1 (Keap1)−Nrf2 (). This makes possible the translocation of Nrf2 to the nucleus of the cell, where it acts as a direct transcriptional factor for the mentioned phase II antioxidant enzymes.[11]

In many publications, the substances of interest are administered in doses too high to be achieved by any food, with the aim of observing a more intense response. As the objective of this study was to evaluate the effect of small quantities of phenolic acids on the activity of phase II enzymes, it was decided to identify a food rich in these substances and administer a single and small quantity of it. It was verified that coffee suits this condition as it has high quantities of caffeic, ferulic, and *p*-coumaric acids.[12,13] Another postive factor in the use of coffee is that it is one of the most popular nonalcoholic beverages in the world.

Therefore, it was decided to evaluate the effect of the administration of a single and small dose of coffee brew to rats, (1) measuring the changes of the hepatic activity of the enzymes SOD, CAT, and GPx; (2) quantifying the extension and characteristics of this response; and (3) determining possible changes in the total antioxidant capacity of the liver tissue.

Received: June 15, 2011
Revised: September 23, 2011
Accepted: September 26, 2011
Published: September 26, 2011

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MATERIALS AND METHODS

Animal Characteristics and Treatment. Male rats (*Ratus novergicus* var. Wistar) with 200 ± 10 g of body weight (BW) were provided by the Animal Facility of the Medical School, University of São Paulo (FM-USP). A total of 48 animals were randomly distributed into 8 groups with 6 animals in each group. During the adaptation period (4 days) and tests, they were kept in individual plastic cages and received purified diet AIN-76 and water ad libitum. The animals were exposed to 12 h light/dark periods and temperature of 22 ± 2 °C. On day 5, they were deprived of food for 12 h before receiving a single dose of coffee brew by gavage. The experimental procedures involving animals were conducted at the Institute of Tropical Medicine (IMT-USP) in compliance with Brazilian laws and approved by the Committee of Ethics in Research of the IMT-USP under no. CEP-IMT-10/07.

Coffee Description and Preparation. Medium roast (degree 3) Brazilian coffee (*Coffea arabica* L. var. Bourbon) cultivated in Minas Gerais and Espírito Santo states and industrialized in 2009, packed under vacuum in 500 g aluminized bags with an external cardboard box, was acquired from local stores. Packs were kept at 4 °C (refrigerator) and in the dark to preserve coffee antioxidant characteristics during the tests (conditions evaluated in a still unpublished study developed by the authors). All tests were done using fresh coffee brews prepared with 80 g of coffee powder per liter of mineral water at 90 °C and filtered through paper filter as recommended by ABIC.[14]

Chemicals. Caffeic, ferulic, and *p*-coumaric acids, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, and fluorescein were acquired from Sigma-Aldrich Co. The commercial kits Ransod and Ransel for SOD and GPx determinations were acquired from Randox Laboratories Ltd. (U.K.). Thirty percent hydrogen peroxide was acquired from Merck KgA (Darmstadt, Germany).

Instrumentation. Determinations of phase II enzyme activities were done using a Shimadzu model 1650 spectrophotometer (Japan). The oxygen radical absorbance capacity (ORAC) tests were done using a Perkin-Elmer model FL-55 fluorimeter (U.K.). Both instruments were equipped with a 10 mm temperature-controlled cell.

Phenolic Acids in Coffee Brew. Phenolic acids are potent bioactive substances capable of increasing the transcription and the activity of phase II antioxidant enzymes.[10,11] As these enzymes are the main concern of this study, one important step was the quantification of caffeic, ferulic, and *p*-coumaric acids in coffee brew. To release these substances from their respective chlorogenic acids (predominant form found in coffee), coffee brew was submitted to an alkaline hydrolysis with 2 N NaOH containing 10 mM of EDTA and 1% of ascorbic acid to prevent the decomposition of phenolic acids.[15] The resulting solution was diluted 1:40 (v/v) with demineralized water, and 20 μL was injected in the HPLC (TSP) fitted with a C18 column (250 × 4.6 mm, 5 μm) using water/methanol/acetic acid (75:24:1 v/v) as mobile phase at 0.8 mL/min.[16] A previous standardization of these methods was done,[13] the quantification was performed using external standard curves, and results were expressed in micrograms per milliliter.

Liver Homogenate Preparation. One gram of washed liver tissue was homogenized with 3 mL of phosphate buffer (0.1 molar, pH 7.0) at 12000 rpm. The sample was centrifuged at 1000*g* for 20 min at 4 °C. The supernatant was collected, transferred to another tube, and centrifuged at 11200*g* for 20 min at 4 °C. The supernatant was collected, diluted 1:10 v/v with phosphate buffer, transferred to another tube, and centrifuged at 30000 rpm for 60 min at 4 °C. The final supernatant was collected and stored at −80 °C in the dark until the end of the analytical determinations (within 48 h from sacrifice).

ORAC. This test was performed by fluorescence spectroscopy (excitation 493 nm, emission 515 nm) using the procedures described by Ou et al.[17] and Prior et al.[18] The test solution was prepared by mixing 2700 μL of phosphate buffer (75 mM, pH 7.4), 300 μL of diluted homogenate (1:40 v/v in phosphate buffer), 15 μL of fluorescein 2.94 mg/L in phosphate buffer, and 300 μL of AAPH 60 g/L in phosphate buffer. The sample was conditioned at 37 °C prior to the addition of the AAPH solution. The fluorescence was recorded every 5 min for 60 min, and the area under the curve (AUC) was calculated using Excel 2007 software. A blank was prepared replacing the sample by 300 μL of phosphate buffer, and a Trolox standard curve was recorded replacing the sample by 300 μL of a 20 μM solution of this substance. Results were expressed as micromoles of Trolox equivalent per liter (μmol TE/L).

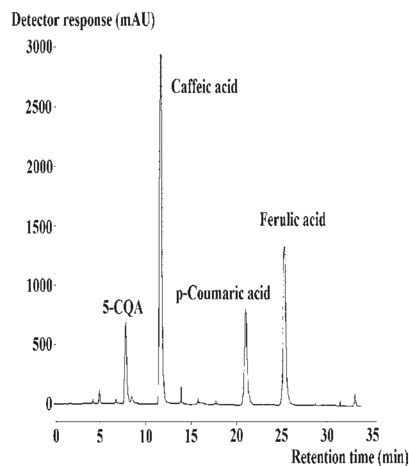

Figure 1. Typical chromatogram obtained during the quantification of phenolic acids present in coffee brew by HPLC.

SOD. The activity of SOD was measured by visible spectroscopy (505 nm) according to the method of Woolliams et al.,[19] modified for the use of the Ransod kit. Thirty-eight microliters of diluted homogenate (1:20 v/v in phosphate buffer 75 mM, pH 7.4) was mixed with 1275 μL of reagent 1 and 187 μL of xanthine oxidase 80 U/L. The temperature was adjusted to 25 °C before the addition of xanthine oxidase, and the absorption was recorded 30 s and 3 min after the addition of the last reagent. Results were expressed in units (U) per milligram of protein.

CAT. The activity of CAT was determined by ultraviolet spectroscopy (230 nm) using the technique described by Aebi.[20] The reaction medium was prepared by mixing 9 mL of H_2O_2 solution (25 μL of 30% H_2O_2 in 50 mL of demineralized water), 0.5 mL of Tris-HCl buffer, and 0.5 mL of demineralized water. Twenty microliters of diluted homogenate (1:5 v/v in phosphate buffer 75 mM, pH 7.4) was added to 980 μL of the reaction medium at 25 °C, and the absorbance was recorded every minute for 5 min. Results were expressed in micromoles per minute per milligram of protein.

GPx. The activity of GPx was determined by ultraviolet spectroscopy (340 nm) using the procedure described by Paglia and Valentine,[21] modified for the use of the Ransel kit. Twenty-five microliters of undiluted homogenate was mixed with 625 μL of reagent 1 and 625 μL of phosphate buffer (75 mM, pH 7.4). The temperature was adjusted to 30 °C, 50 μL of cumene hydroperoxide 0.18 mM was added, and the absorbance was recorded 1, 2, and 3 min after the addition of the last reagent. Results were expressed in units per milligram of protein.

Statistical Calculations. Statistical calculations (Kolmogorov–Smirnov, Student's *t* test, and Pearson's correlation test) were performed using the SPSS 16.0 for Windows software package, with a significance level of $p < 0.05$. All tests were done in triplicate, and the results are presented as the mean ± standard deviations.

Table 1. Comparison of Individual and Total Phenolic Acids Found in Coffee Brews of Different Studies and the Respective Coffee Powder/Water Ratio Used To Prepare the Infusions

substance	present study	ref 22	ref 23	ref 24
caffeic acid (μg/mL)	685.1 ± 30.8	960.0 ± 31.3	830.0 ± 69.9	870.0 ± 22.0
ferulic acid (μg/mL)	97.9 ± 8.0	90.0 ± 3.0	142.8 ± 12.3	91.0 ± 3.6
p-coumaric acid (μg/mL)	10.3 ± 1.2	13.7 ± 0.6	14.0 ± 1.1	12.7 ± 0.5
Σ phenolic acids (μg/mL)	793.3	1,063.7	986.8	973.7
coffee/H_2O (g/L)	80	54	60	50

RESULTS AND DISCUSSION

Phenolic Acids in Coffee Brew. After the chromatographic tests (Figure 1), coffee brew showed high levels of caffeic, ferulic, and *p*-coumaric acids, confirming that this beverage was a good choice to study the increase of phase II enzymes through a nutrigenomic model. The sum of these compounds totaled 793.3 μg/mL of coffee brew, as shown in Table 1. This amount showed to be from 22.7 to 34.1% lower than the results obtained in similar studies with coffee brews,[22−24] even using a higher coffee powder/water ratio during the preparation of the infusion (Table 1). Besides the differences between brewing methods, a possible explanation would be the mild Brazilian climate, given that phenolic acids are secondary metabolites, generally involved in plant adaptation or defense against environmental stressful conditions, as stated by Farah and Donangelo.[25] The results indicated that a single dose of 2 mL of coffee brew administered to rats with 200 g of BW would represent a dose of 8 mg of total phenolic acids/kg of BW that fits very well the purpose of this study to administera small doses of these substances.

Time of Response. The first set of experiments evaluated the time required to change the activities of the hepatic enzymes SOD, CAT, and GPx after the administration of a single dose of coffee brew. After fasting, group 1 (control) received 2 mL of mineral water. One hour after the gavage, the animals were anesthetized and sacrificed, and the liver tissues were washed with sterile 9 g/L sodium chloride solution and frozen in liquid nitrogen to preserve them until the preparation of the homogenates (maximum 2 h after the sacrifice). The average activities of the antioxidant enzymes of this group were SOD = 13.6 ± 1.6 U/mg of protein, CAT = 8.6 ± 1.2 μmol/min·mg of protein, and GPx = 18.3 ± 2.4 U/mg of protein, well-suited to the results obtained in other studies with similar animal models.[10,26−28]

Groups 2−5 were submitted to the same conditions as group 1 replacing the mineral water by 2 mL of coffee brew, and the sacrifice was done 1, 2, 3, and 4 h after the gavage, respectively. All three enzymes presented a peak of activity 1 h after the gavage (group 2), which decreased from this time on (Figure 2). Even 4 h after the gavage (group 5), the activities did not return to the basal figures, suggesting a long-term effect. At the maximum activity time (1 h), SOD, CAT, and GPx average activities increased to 16.2 ± 1.5 U/mg of protein, 10.5 ± 0.9 μmol/min·mg of protein, and 22.9 ± 2.3 U/mg of protein, respectively, which means increases of 19.1, 22.1, and 25.1% in comparison to the control group. The differences between group 1 (basal) and group 2 (maximum activity) were statistically significant (p = 0.015 for SOD and CAT, p = 0.007 for GPx), which represents an interesting result considering the low exposure to coffee brew (single dose).

Working with pure phenolic acids dissolved in propylene glycol, Yeh and Yen[10] observed statistically significant increases

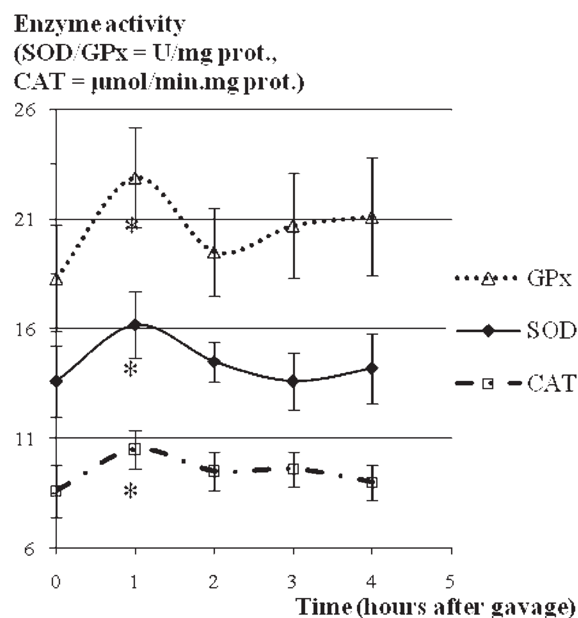

Figure 2. SOD (U/mg of protein), CAT (μmol/min·mg of protein), and GPx (U/mg of protein) activities in the liver tissue of rats (mean ± standard deviation) versus time for the sacrifice after 2 mL of coffee brew (n = 6). Time zero represents control group results. (* = different from control group at $p < 0.05$.)

in the activities of SOD, CAT, and GPx in the hepatic tissue of rats. In their study, each animal received 100 mg of different phenolic acids/kg of BW for 14 consecutive days. The given dose was equivalent to 50 medium cups of coffee per day for a human with 70 kg of BW, and the use of phenolic acid supplements was the only alternative to achieve it. The present work also showed statistically significant changes, but they were obtained after a single and much smaller dose of phenolic acids present in 2 mL of coffee brew (8 mg of total phenolic acids/kg of BW). This volume is equivalent to 4 medium cups of coffee for a human with 70 kg of BW, which represents a reasonable quantity of this beverage. Therefore, the results were obtained without overexposing the animals to the substances of interest, and they suggest that the threshold to observe a significant change in the activity of these enzymes is actually much lower than the data previously published.[10] Furthermore, the present study measured the residual effect until 4 h after the administration, suggesting a long-term enzymatic change before a return to basal levels. In addition, a concomitant increase in the activities of SOD, CAT, and GPx was identified that highlights the interesting alternative found by the evolution as these enzymes work in a chain-type process. This fact avoids the excessive

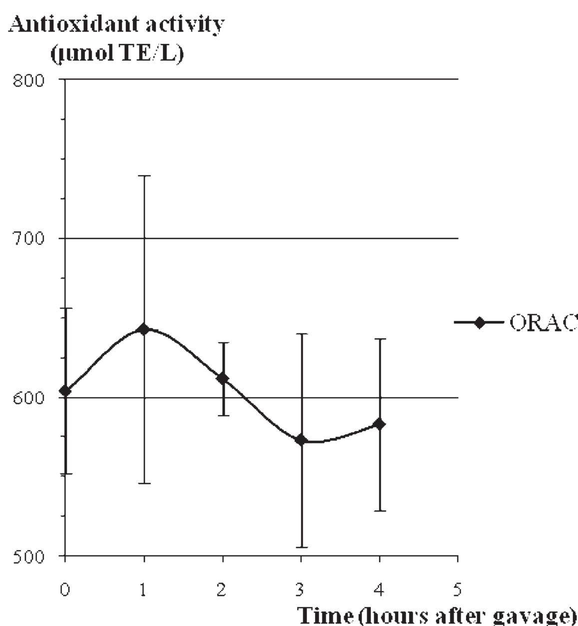

Figure 3. Total antioxidant activity measured by ORAC (μmol TE/L) in the liver tissue of rats (mean ± standard deviation) versus time for the sacrifice after 2 mL of coffee brew (n = 6). Time zero represents control group result.

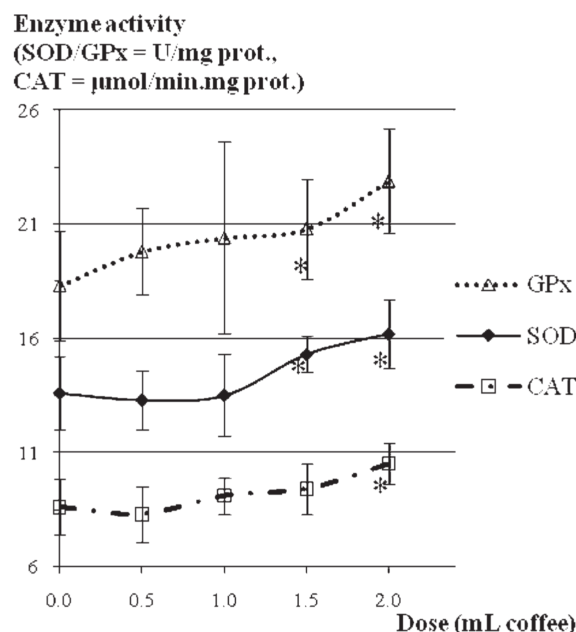

Figure 4. SOD (U/mg of protein), CAT (μmol/min·mg of protein), and GPx (U/mg of protein) activities in the liver tissue of rats (mean ± standard deviation) versus volume of coffee brew (n = 6). Volume zero represents control group results. (∗ = different from control group at $p < 0.05$.)

concentration of any dangerous intermediate substance during enzymatic detoxification.

Using the same biological samples, the total antioxidant capacity of the liver tissue was measured by ORAC. The control group showed an average result equal to 604 ± 52 μmol TE/L that increased to 643 ± 97 μmol TE/L in group 2, decreasing from this time on (Figure 3). ORAC average values also showed a peak 1 h after the gavage, in a similar profile when compared to the enzymes SOD, CAT, and GPx, but the magnitude of the ORAC changes was less intense. This observation can be justified by the fact that the total antioxidant capacity of the hepatocytes is the sum of the action of several antioxidant agents that includes, but it is not limited to, the phase II enzymes. Another important fact to be mentioned is that antioxidant molecules such as phenolic acids present in large quantities in coffee brew can increase ORAC results. Therefore, the observed effect must not be assigned exclusively to the increase of the enzymes activities. The difference between group 1 (control) and group 2 (maximum effect) was not statistically significant (p = 0.403), probably due to the low exposure to coffee brew (single dose). Yeh and Yen[10] observed statistically significant increases of the ORAC average values, but the animals were exposed to a much higher dose of phenolic acids during 14 consecutive days. Still unpublished data obtained by the authors showed that the difference between a group receiving 2 mL of coffee brew per day over 30 consecutive days and its respective control group was statistically significant ($p < 0.001$), suggesting a cumulative effect over a long-term exposure.

It was also noted that the ORAC average values obtained in groups 4 and 5 were below the control group value, with a tendency to return to basal line later than 4 h after the intervention. This fact was also observed in other published studies that have evaluated the variation of ORAC after the administration of different antioxidant foods.[18,29] A possible explanation would be the overproduction of the peroxide ion (O_2^{2-}) by the increased activity of SOD[8] or by the intracellular homeostasis based mainly on the reduced/oxidized glutathione pair (GSH/GSSG) and thioredoxin (TRx) that would be acting to maintain the redox state inside the hepatocytes.[6,30]

Therefore, after this first set of experiments it was possible to conclude that a single administration of 2 mL of coffee brew was enough to increase the activity of hepatic SOD, CAT, and GPx, the maximum transitory change occurred 1 h after the gavage, and the effect started to decrease from this time on without returning to basal levels even 4 h after the intervention. The total hepatic antioxidant capacity also increased under the same conditions, the maximum effect occurred 1 h after the intervention, but the changes were not statistically significant due to the low exposure to coffee.

Dose × Response. The second set of experiments evaluated the relationship between the volume of coffee brew given in a single administration and the changes of SOD, CAT, and GPx average activities. Groups 6, 7, and 8 received 0.5, 1.0, and 1.5 mL of coffee brew, respectively, and the sacrifice of all groups occurred 1 h after the gavage (maximum activity time identified in the previous item). The results obtained in groups 1 (2 mL of water) and 2 (2 mL of coffee brew) were reused to avoid unnecessary sacrifice of animals.

For SOD, doses between 0.0 and 2.0 mL of coffee brew resulted in increasing average activities from 13.6 ± 1.6 to 16.2 ± 1.5 U/mg of protein (Figure 4). Groups 8 and 2 that received greater volumes of coffee brew (1.5 and 2.0 mL) showed statistically significant differences from control group (p = 0.038 for group 8, p = 0.015 for group 2), whereas groups 6 and 7 that received smaller doses (0.5 and 1.0 mL) did not present statistically significant differences (p = 0.972 for group 6, p = 0.974 for group 7). Pearson's test for the variables volume of coffee brew and SOD average activity showed a linear, positive, and statistically significant correlation equal to 0.879 (p = 0.049).

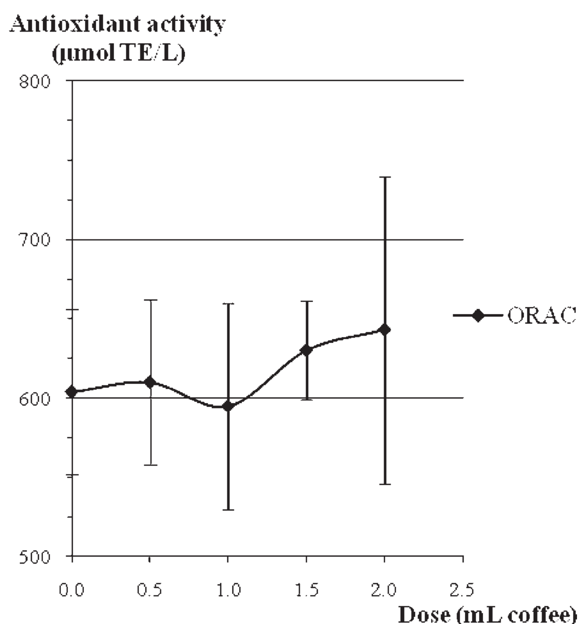

Figure 5. Total antioxidant activity measured by ORAC (μmol TE/L) in the liver tissue of rats (mean ± standard deviation) versus volume of coffee brew (n = 6). Volume zero represents control group result.

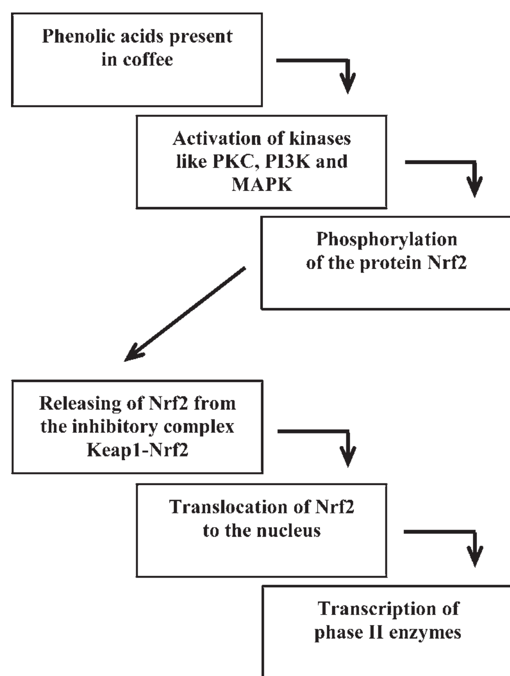

Figure 6. Proposed mechanism for the indirect action of phenolic acids present in coffee brew on the transcription of phase II enzymes.

For CAT, the same volumes of coffee brew resulted in average activities between 8.6 ± 1.2 and 10.5 ± 0.9 μmol/min·mg of protein (Figure 4). Despite the increase of CAT activity with larger volumes of coffee, only group 2 (2 mL) showed a statistically significant difference from the control group ($p = 0.657$ for group 6, $p = 0.437$ for group 7, $p = 0.285$ for group 8, $p = 0.015$ for group 2). A linear, positive, and statistically significant correlation between volume of coffee brew and CAT average activity equal to 0.909 was observed by the application of the Pearson's test ($p = 0.033$).

Under the same conditions, GPx showed average results between 18.3 ± 2.4 and 22.9 ± 2.3 U/mg of protein (Figure 4). The differences from the control group were statistically significant in groups 8 ($p = 0.043$) and 2 ($p = 0.007$), which received greater volumes of coffee, and not significant in groups 6 ($p = 0.254$) and 7 ($p = 0.313$). Likewise for SOD and CAT, Pearson's test showed a linear, positive, and statistically significant correlation equal to 0.965 between volume of coffee brew and GPx average activity ($p = 0.008$).

The same biological samples were used to evaluate the relationship between volume of coffee brew and total antioxidant capacity of liver tissue (ORAC). The previously mentioned volumes produced increasing average results from 604 ± 52 to 643 ± 97 μmol TE/L (Figure 5), but the differences between all groups exposed to coffee brew and control group were not statistically significant ($p = 0.833$ for group 6, $p = 0.800$ for group 7, $p = 0.311$ for group 8, $p = 0.403$ for group 2), probably due to the low exposure to coffee already mentioned. Pearson's test showed a linear, positive, but not statistically significant correlation equal to 0.788 between volume of coffee brew and ORAC ($p = 0.113$).

In addition to the conclusions already discussed under Time of Response, these results demonstrated that the activity of the enzymes SOD, CAT, and GPx increased after the administration of small volumes of coffee, and the changes presented a dose-dependent characteristic at a significance level of $p < 0.05$. For a long time, coffee has been considered an antioxidant beverage due to several molecules present in the fruit, such as phenolic compounds and caffeine, or developed during roasting, such as melanoidins and Maillard reaction products. As phase II enzymes act in defense against oxidative stress, quenching mainly the primary radicals superoxide (by SOD) and peroxide (by CAT and GPx), the findings of this study indicate that coffee can additionally act as an antioxidant beverage through another pathway, supplying indirect transcriptional factors for the modulation of phase II antioxidant enzymes (Figure 6) and/or directly activating the mentioned enzymes, increasing their activities. In both cases, the organism would be better protected against oxidative stress and its catastrophic consequences. As previously published,[10,11] the increasing activities of SOD, CAT, and GPx in the hepatic tissue after the administration of phenolic acids are regulated on a transcriptional level.

The conclusions of the present study strongly suggest the development and publication of a long-term, low-exposure experiment to authenticate these changes under a repetitive exposure condition.

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Funding Sources

This work was financially supported by the State of São Paulo Research Foundation (FAPESP) and the National Council for Scientific and Technological Development (CNPq).

ABBREVIATIONS USED

SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; Nrf2, nuclear factor-E2-related factor; Keap1, Kelch-like ECH-associated protein 1; AAPH, 2,2′-azobis(2-amidinopropane)

dihydrochloride; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Tris-HCl buffer, tris(hydroxymethyl)aminomethane hydrochloride; ORAC, oxygen radical absorbance capacity; TE, Trolox equivalent.

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