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Research Article

Effect of coffee Melanoidin on human hepatoma HepG2 cells. Protection against oxidative stress induced by *tert*-butylhydroperoxide

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Soluble high-molecular weight fraction (named melanoidin) from coffee brew was isolated by ultrafiltration, subsequently digested by simulating a gastric plus pancreatic digestive condition and partly characterized by CZE, gel-filtration and browning. The objective of the present study was to investigate the potential protective effect of the coffee melanoidin submitted to gastrointestinal digestion on cell viability (lactate dehydrogenase leakage) and redox status of cultured human hepatoma HepG2 cells submitted to oxidative stress induced by *tert*-butylhydroperoxide (*t*-BOOH). Concentration of reduced glutathione (GSH) and malondialdehyde (MDA), generation of reactive oxygen species (ROS) and activity of antioxidant enzymes glutathione peroxidase (GPx) and reductase (GR) were used as markers of cellular oxidative status. Pretreatment of cultured HepG2 cells with 0.5–10 μg/mL digested coffee melanoidin (DCM) for 2 or 20 h completely prevented the increase in cell damage and GR and partly prevented the decrease of GSH and the increase of MDA and GPx evoked by *t*-BOOH in HepG2 cells. In contrast, increased ROS generation induced by *t*-BOOH was not prevented when cells were pretreated with DCM. The results show that treatment of HepG2 cells with concentrations of DCM within the expected physiological range confers the cells a significant protection against an oxidative insult.

Keywords: Antioxidant defences / Biomarkers for oxidative stress / Coffee melanoidin / Dietary antioxidants / Maillard reaction products

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1 Introduction

Maillard reaction (MR) takes place during the thermal processing of foodstuffs by condensation between the amino groups of a free or protein-bound amino acid and reducing sugars. The high-molecular weight compounds formed at the late stage of the MR during home- and industrial processing of foods are named melanoidins and are widely distributed in our diet. As reviewed by Martins and van Boekel [1], three main proposals for the structure of melanoidins have been put forward: (i) low-molecular weight coloured substances crosslinked to free amino groups of lysine or arginine in proteins [2], (ii) units of furan and/or pyrroles that, through polycondensation reactions, form melanoidin repeating units [3], (iii) melanoidin skeleton is mainly built up from sugar degradation products formed in the early stages of the MR, polymerized and linked by amino compounds [4]. Recently, some authors suggested that coffee melanoidins may contain phenolic subunits [5].

Melanoidins have been studied in recent years due to their nutritional, biological and health implications apart from their well-known technological and organoleptic implications on the overall food acceptability by consumers [6–9]. Briefly, melanoidins possess antioxidant [10, 11] and antimicrobial activity [12], contribute to food texture and are responsible for the development of colour in heat-processed food products [7]. In addition, melanoidins exert anticariogenic [13] and antitumoural properties [14], as well as prebiotic effects [15].

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Abbreviations: DCFH, dichlorofluorescin; **DCM,** digested coffee melanoidin; **FBS,** foetal bovine serum; **GPx,** glutathione peroxidase; **GR,** glutathione reductase; **GSH,** glutathione; **LDH,** lactate dehydrogenase; **MDA,** malondialdehyde; **MR,** Maillard reaction; **MRP,** Maillard reaction product; **ROS,** reactive oxygen species; t**-BOOH,** *tert*-butylhydroperoxide

The antioxidant activity of melanoidins is especially interesting since these products are naturally formed during the food processing and storage and they can influence the oxidative and shelf life of foods, such as cereal [16], milk [17], coffee [18], meat [19] and tomato juice [20]. This melanoidin effect has been reported to be due to their effect as metal chelating agents [21], and their peroxyl or hydroxyl radical scavenging activity [22, 23]. In line with their antioxidant activity, some physiological effects of melanoidins have been stated such as induction of the chemopreventive enzyme system [7, 24] and their protective effect against lipid peroxidation in isolated rat hepatocytes [25].

A high proportion of daily melanoidins intake comes from coffee beverage (up to 30% coffee dry matter), antioxidant properties of which have frequently been attributed to certain phenolic compounds present in the green coffee, such as chlorogenic acid, caffeic acid, ferulic acid or *p*-coumaric acid. The roasting process affects the final composition of coffee and levels of naturally occurring phenolic acids decrease depending on the severity of roasting conditions. Therefore, although the effect of polyphenolic compounds cannot be ruled out, melanoidins seem to be the prevailing contributors to the maintained antioxidant activity of coffee brew [5, 26].

This research aimed to investigate the protective effect of food melanoidins isolated from roasted coffee and subsequently treated to simulate a gastrointestinal digestion. A cell model of human hepatoma HepG2 cells [27] was used for assessing the protection of digested coffee melanoidin (DCM) against oxidative stress.

2 Materials and methods

2.1 Cell culture

Human hepatoma HepG2 cells were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37°C. They were grown in DMEM F-12 medium from Biowhitaker (Innogenetics, Madrid, Spain), supplemented with 2.5% Biowhitaker foetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin (all from Sigma, Madrid, Spain). Plates were changed to FBS-free medium before the beginning of the assay. The serum added to the medium favours growth of most cell lines but might interfere in the running of the assays and affect the results. Moreover, a fairly good growth of HepG2 cells was observed in FBS-free DMEM-F12 [28].

2.2 Isolation, purification, analysis and digestion of melanoidins from coffee brew

Coffee brew was prepared from highly roasted (18.9% roasting loss) coffee beans (blend of 80% Arabica and 20% robusta). Melanoidins were obtained after ultrafiltration (10 kDa cut-off) of coffee brew as previously described by Delgado-Andrade and Morales [26]. Analysis of melanoidins by High-Performance Gel Permeation Chromatography (HPGPC) and further analysis by CZE were performed as described by Morales [29]. Figure 1 depicts some chromatographic profiles for coffee brew (a), high-molecular weight fraction (b) and low-molecular weight fraction obtained from coffee brew (c). Simulated gastrointestinal digestion of coffee melanoidins was carried out by following the method described by Ames *et al.* [30]. Briefly, a sample (0.5 g) was diluted with distilled water (4 mL). After adding freshly prepared pepsin (3.2 g/20 mL 0.1 M

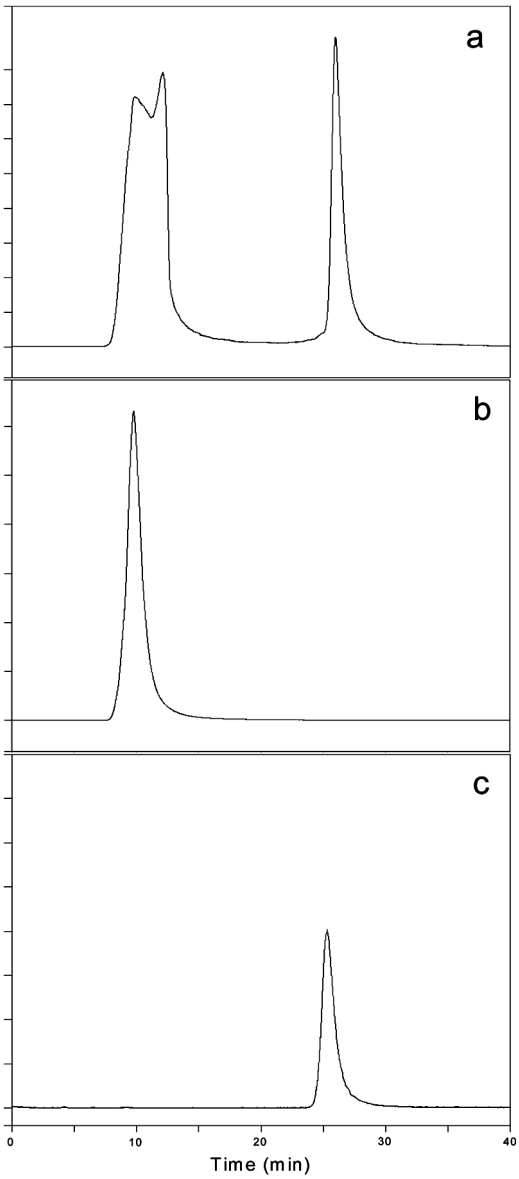

Figure 1. (a) Chromatographic profiles for coffee brew, (b) coffee melanoidin and (c) low-molecular weight fraction after filtration. Samples (20 μL) eluted in water at 0.8 mL/min and recorded at 280 nm.

HCl), the pH was adjusted to 2.0 using 6 M HCl, and the mixture was incubated at 37°C in a shaking water bath. After the gastric digestion step, the sample was adjusted to pH 7.0 using 0.5 M $NaHCO_3$ and 1.25 mL of freshly prepared pancreatinin–bile mixture (0.4 g pancreatinin, and 2.5 g bile salts in 100 mL 0.1 M $NaHCO_3$) before incubating 1 h at 37°C in a shaking water bath. At the end of the gastrointestinal digestion, the sample was heated in a boiling bath for 4 min in order to inactivate the enzymes that could cause interferences with cell experiments. The sample was lyophilized and stored at −20°C until analysis.

2.3 Cell treatment

Two different types of experiments were designed for this study: (A) experiments of plain treatment of cells with DCM for 4 and 24 h to test for a direct effect of the DCM and (B) experiments of pretreatment of cells with DCM for 2 or 20 h before submitting the cells to an oxidative stress by *tert*-butylhydroperoxide (*t*-BOOH) to test for a protective effect of the melanoidin against an oxidative insult. Lactate dehydrogenase (LDH), glutathione (GSH), malondialdehyde (MDA) and reactive oxygen species (ROS) were evaluated in both experimental conditions and, in addition, glutathione peroxidase (GPx) and glutathione reductase (GR) were also determined in experiment B. In the experiments of plain treatment (experiment A), the different concentrations of DCM, dissolved in serum-free culture medium, were added to the cell plates for 4 (short term) or 24 (long term) h. Then, cell culture was collected (LDH assay) or eliminated (GSH and MDA assays) and the cells washed with PBS, collected by scraping and treated as described below for each assay. In the ROS assay for experiment A, cells cultured in 24-well multiwell plates were treated with DCM as above, the dichlorofluorescin (DCFH) probe was added for 30 min, then they were washed twice before being treated with plain medium (DCM-free) and with (*t*-BOOH wells) or without (the rest of wells) 200 μM *t*-BOOH for 90 min. In the ROS assay for experiment B, the different concentrations of DCM were added to the cell plates for 2 (short term) or 20 (long term) h, the DCFH probe added for 30 min, and the cell plates were washed twice with PBS and new DCM-free medium containing 200 μM *t*-BOOH was added to all cultures except controls for 90 min of the assay. The selection of the concentrations of DCM to test is explained below (see Discussion).

2.4 Evaluation of cytotoxicity (LDH leakage assay), reduced GSH and MDA

Cells were plated in 60 mm diameter plates at a concentration of 1.5×10^6 per plate and the assay was carried out two days later. Cells were treated as described in the section of melanoidin treatment and LDH leakage to the culture medium was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content [27, 28, 31]. The content of reduced GSH was quantitated by the fluorometric assay of Hissin and Hilf [32]. The method takes advantage of the reaction of reduced GSH with *o*-phthalaldehyde (OPT) at pH 8.0. Fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The precise protocol has been described elsewhere [27, 28, 31]. Cellular MDA was analysed by HPLC as its 2,4-dinitrophenylhydrazone (DNPH) derivative [33]. Cells were treated as in the LDH assay and then collected. Values are expressed as nmol of MDA/mg protein; protein was measured by the Bradford method [34].

2.5 Determination of ROS

Cellular ROS were quantified by the DCFH assay using microplate reader [35]. After being oxidized by intracellular oxidants, DCFH will become DCF and emit fluorescence. By quantifying fluorescence over a period of 90 min, a fair estimation of the overall oxygen species generated under the different conditions was obtained. The assay has been described elsewhere [27, 28, 31].

2.6 Determination of GPx and GR activity

For the assay of the GPx and GR activity, cells previously treated as in LDH, reduced GSH and MDA assays were suspended in PBS and centrifuged at low speed for 5 min to pellet cells. Cell pellets were resuspended in 20 mM Tris, 5 mM EDTA and 0.5 mM mercaptoethanol, sonicated and centrifuged at $3000 \times g$ for 15 min. The enzyme activity was measured in the supernatants. The determination of GPx activity is based on the oxidation of reduced GSH by GPx, using *t*-BOOH as a substrate, coupled to the disappearance of NADPH by GR [36]. GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized GSH [37]. The methods have been previously described [27, 28, 31]. Protein was measured by the method of Bradford [34].

2.7 Statistics

Statistical analysis of data was as follows: prior to analysis the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by Tamhane test when variances were not homogeneous. The level of significance was $p < 0.05$. An SPSS version 12.0 program has been used.

3 Results

3.1 Experiment A: Plain treatment of HepG2 with DCM

3.1.1 Cytotoxicity

LDH leakage was used as an indicator of cytotoxicity. In a preliminary study, a range of doses between 0.5 and 100 μg/mL was tested for periods of 4 and 24 h for their potential deleterious effect on HepG2 cells, but only results of the four selected concentrations (0.5 – 10 μg/mL) are presented (% LDH activity in the culture medium after 24 h with 50 and 100 μg/mL was 6.8 ± 0.4 and 5.4 ± 0.6, respectively). Figure 2a shows that no increase in LDH leakage to the culture medium was observed with any concentration of DCM after a treatment for 4 or 24 h. Control values for both assays, 4 and 24 h, were pooled for the homogeneity of results. A batch of cultured cells was treated with 200 μM *t*-BOOH for 3 h as a positive control for severe cell damage and increased LDH leakage and the results are shown within the same figure for comparison.

3.1.2 Concentration of GSH

As an index of the intracellular nonenzymatic antioxidant defences, the concentration of reduced GSH was measured in HepG2 cells treated with increasing concentrations of DCM. As a positive control for a prooxidative status and GSH utilization, some cell plates were exposed to 200 μM *t*-BOOH for 3 h and the results are shown in the same figure for comparison (Fig. 2b). Control values for both assays, 4 and 24 h, were pooled for the homogeneity of results. A significant 80% decrease in GSH was observed in cells treated with *t*-BOOH. No changes in the intracellular level of GSH were observed in HepG2 treated with a range of DCM between 0.5 and 10 μg/mL for 4 h, whereas a dose-dependant reduction in GSH concentration was observed after 24 h, reaching 50% of control values at 10 μg/mL.

3.1.3 MDA levels

As a biomarker for lipid peroxidation, the cytoplasmic concentration of MDA was measured. A 3 h treatment of HepG2 with 200 μM *t*-BOOH evoked a significant increase of about 50% in the cellular concentration of MDA, indicating oxidative damage to cell lipids (Fig. 2c). When HepG2 cells were treated for 4 h with all four concentrations of DCM, no changes in MDA concentration were observed. In contrast, a decrease in MDA was found at 1 and 10 μg/mL that became statistically significant in cells treated with 5 μg/mL DCM for 24 h (Fig. 2c).

3.1.4 ROS generation

As a positive control for ROS production, some HepG2 cell plates were treated for the length of the assay (90 min) with 200 μM *t*-BOOH and a significant increase in ROS generation was observed over time in the presence of the stressor as compared to nonstressed controls (Fig. 3). Cells were treated for 4 (Fig. 3a) or 24 h (Fig. 3b) with the coffee melanoidin, incubated for 30 min with the fluorescent probe (DCFH), washed out and the fluorescent emission evaluated for 90 min. In both cases, 4 and 24 h, cells treated with 0.5 – 10 μg/mL DCM generated ROS up to levels that were in the range of those of control nonstressed cells. Due to interassay variation of fluorescent emission, only results obtained within the same DCF assay have been compared.

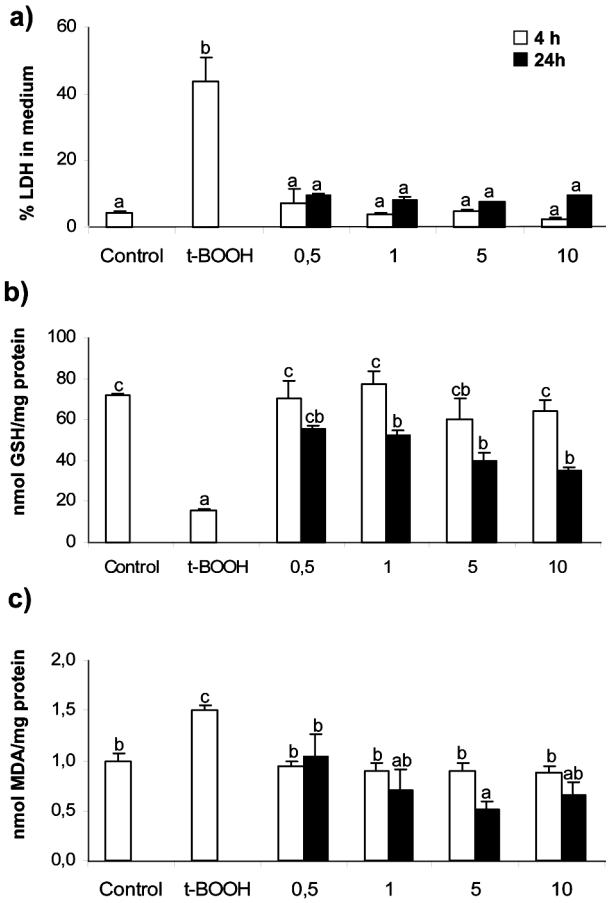

Figure 2. Experiment A: Effect of DCM on cell viability and intracellular concentration of reduced GSH and MDA. HepG2 cells were treated with the noted μM concentrations of DCM for 4 or 24 h. Results from 4 and 24 h were pooled in a single bar both for control and *t*-BOOH data. As a positive control for LDH leakage, GSH depletion and MDA generation, a batch of cultures (noted as *t*-BOOH) was treated with 200 μM *t*-BOOH for 3 h. (a) Results of LDH leakage are expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular. Values are means ± SD of 6 – 8 data. (b) The results of the fluorescent analysis for reduced GSH, are means of 4 – 5 different samples per condition. (c) Quantitative analysis of MDA was made by HPLC in cytoplasmatic contents of HepG2. Values are means ± SD, $n = 4$. Different letters indicate statistically significant differences ($p < 0.05$) among different groups.

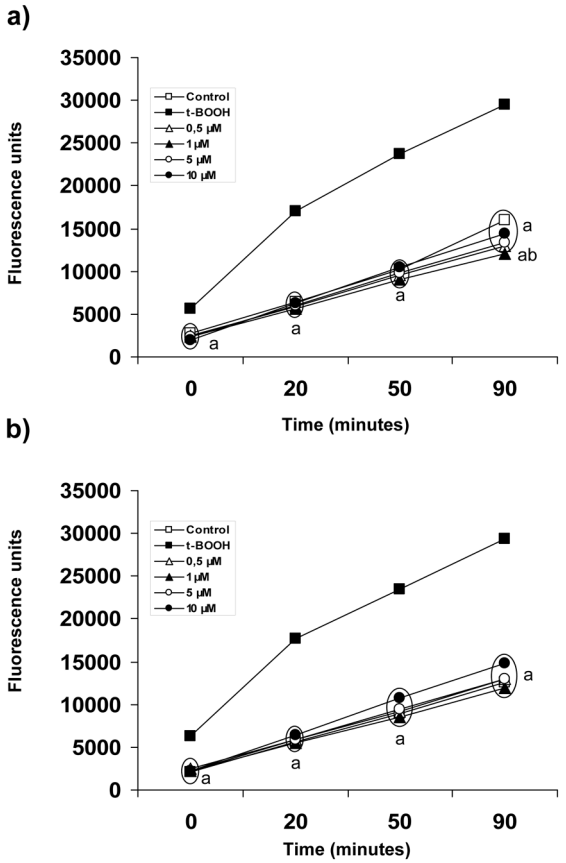

Figure 3. Experiment A: Effect of DCM on intracellular ROS generation. HepG2 cultures were treated with the noted μM concentrations of DCM for 4 (a) or 24 (b) h; as a positive control for enhanced ROS generation a batch of cultures (noted as *t*-BOOH) was treated with 200 μM *t*-BOOH for the length of the assay. The results are expressed in fluorescence units. Different letters upon symbols indicate statistically significant differences ($p < 0.05$) when that group or close groups of data are compared to *t*-BOOH (letter a) or to control (letter b). Values are means ± SD of 7–8 different samples per condition. SD values were not included due to intense bar overlapping.

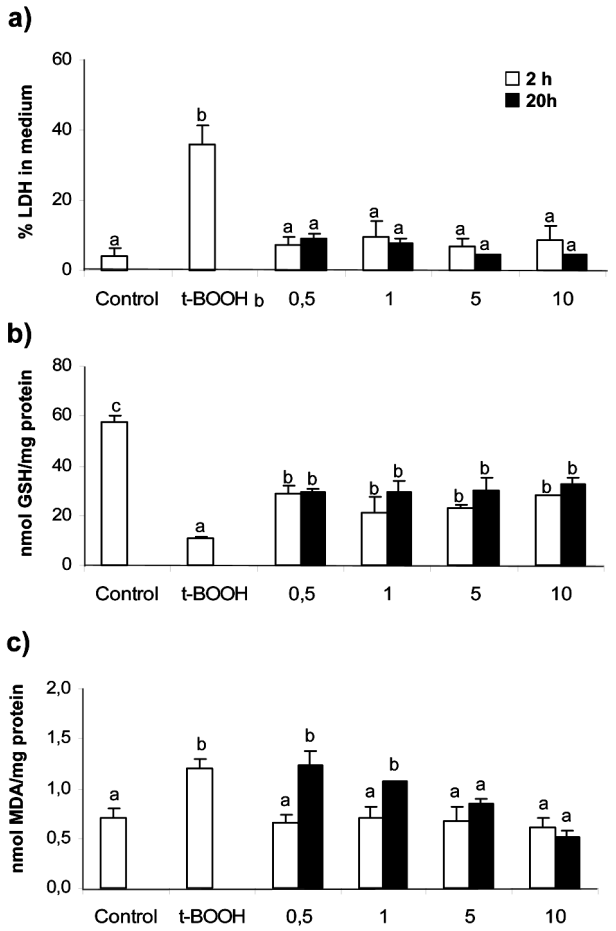

Figure 4. Experiment B: Protective effect of DCM against oxidative stress on cell viability and intracellular concentration of reduced GSH and MDA. HepG2 cells were treated with the noted μM concentrations of DCM for 2 or 20 h, then the cultures were washed and 200 μM *t*-BOOH was added to all the cultures except controls for 3 h. Results from 2 and 20 h were pooled in a single bar both for control and *t*-BOOH data. (a) Results of LDH leakage are expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular. Values are means ± SD of 6–8 data. (b) The results of the fluorescent analysis for reduced GSH are means of 4–5 different samples per condition. (c) MDA in cytoplasmatic contents of HepG2 are means ± SD, $n = 4$. Different letters indicate statistically significant differences ($p < 0.05$) among different groups.

Leakage of probe was not observed in cells throughout the assay, as determined in our laboratory in previous tests during method setup [27]. Thus, any potential contribution of extracellularly oxidized DCF to the final fluorescence can be ruled out.

3.2 Experiment B: Pretreatment of HepG2 with DCM before exposure to *t*-BOOH

Once stated that the concentrations of DCM used on HepG2 in culture were not cytotoxic and did not significantly affect the cellular basal redox balance, the potential protective effect of DCM in conditions of oxidative stress induced by a potent prooxidant, *t*-BOOH, was tested.

3.2.1 Cytotoxicity

In our experimental conditions treatment with 200 μM *t*-BOOH for 3 h evoked a great increase in LDH activity in the cell culture medium indicating cell damage in HepG2 (Figs. 2a and 4a). Pretreatment for 2 or 20 h of HepG2 cultures with 0.5–10 μg/mL DCM prevented cell damage induced by *t*-BOOH, maintaining LDH ratios in the range of control nonstressed cells.

3.2.2 GSH concentration

The concentration of reduced GSH was measured in cells treated with *t*-BOOH which had been pretreated for 2 or 20 h with 0.5 – 10 μg/mL DCM. *t*-BOOH (200 μM) evoked a dramatic decrease of cytoplasmic GSH which was partly overcome by a pretreatment for 2 or 20 h with any of the above doses of DCM (Fig. 4b). A recovery of about 50% of the control values was observed in all conditions.

3.2.3 MDA levels

A short-term pretreatment of 2 h with all four doses of DCM completely prevented the MDA increase induced by a treatment with 200 μM *t*-BOOH for 3 h, indicating a reduced level of lipid peroxidation (Fig. 4c). However, following a pretreatment of 20 h with DCM, a dose-dependant response was observed and the two highest concentrations, 5 and 10 μg/mL significantly prevented the increased MDA in response to *t*-BOOH (Fig. 4c).

3.2.4 ROS generation

Cells were treated for 2 or 20 h with the same doses of DCM and incubated with the fluorescent probe (DCFH) for 30 min, then, the culture medium with the coffee melanoidin and the probe was removed and 200 μM *t*-BOOH were added for the length of the ROS assay, 90 min. As in the case of experiment A, a significant increase in ROS production was observed over time in the presence of 200 μM *t*-BOOH as compared to nonstressed controls (Fig. 5). Pretreatment for either 2 (Fig. 5a) or 20 h (Fig. 5b) of HepG2 cultures with 0.5 – 10 μg/mL DCM did not prevent ROS production induced by *t*-BOOH (Fig. 5) which was similar in all cells submitted to *t*-BOOH, pretreated or not with DCM.

3.3 Activity of GPx and GR

The presence of 200 μM *t*-BOOH in the culture medium for 3 h induced a significant increase in the enzyme activity of GPx and GR (Fig. 6). The response of GPx to *t*-BOOH was dependent on the time of DCM pretreatment. The activity of GPx in cells pretreated 2 h with 0.5 – 10 μg/mL DCM and submitted to *t*-BOOH was similar to that of cells only submitted to the *t*-BOOH, whereas when cells were pretreated for 20 h with the same range of doses of DCM, the *t*-BOOH-induced increase in GPx activity was totally suppressed. A pretreatment of cells with all four doses of DCM for either 2 or 20 h completely prevented the increase in GR induced by *t*-BOOH.

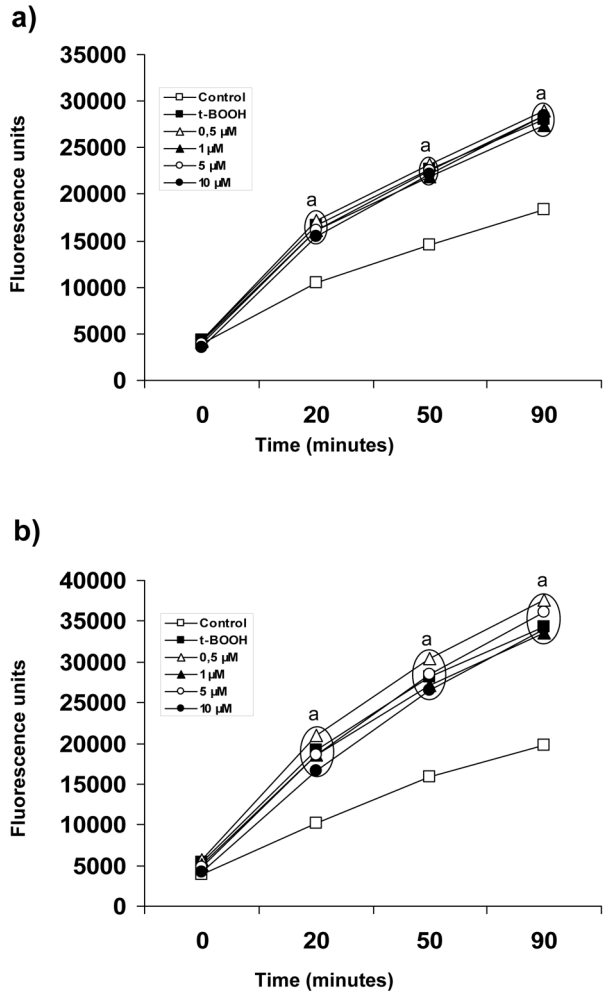

Figure 5. Experiment B: Protective effect of DCM against oxidative stress on intracellular ROS generation. HepG2 cultures were treated with the noted μM concentrations of DCM for 2 h (a) or 20 h (b), then the cultures were washed and 200 μM *t*-BOOH was added to all cells except controls and intracellular ROS production was evaluated at 0, 20, 50 and 90 min and expressed as fluorescence units. Letter a upon symbols indicates statistically significant differences ($p < 0.05$) when that group or close groups of data are compared to control. Values are means ± SD of 7 – 8 different samples per condition. SD values were not included due to intense bar overlapping.

4 Discussion

There is considerable current interest in the cytoprotective effects of dietary compounds against oxidative stress and the different defence mechanisms involved. Dietary antioxidants may protect against oxidative stress, and human diet contains antioxidants that are formed in heat-treated foods. During food processing and storage, chemical reactions among food components lead to the formation of secondary antioxidants that may play a role in protection against cell oxidative damage [38]. Maillard reaction products (MRPs) are important candidates for food antioxidants and, therefore, have been investigated for nutritional, physiological and biological activities [39]. Although the exact nature of the products formed is not yet well known, high-molecular-

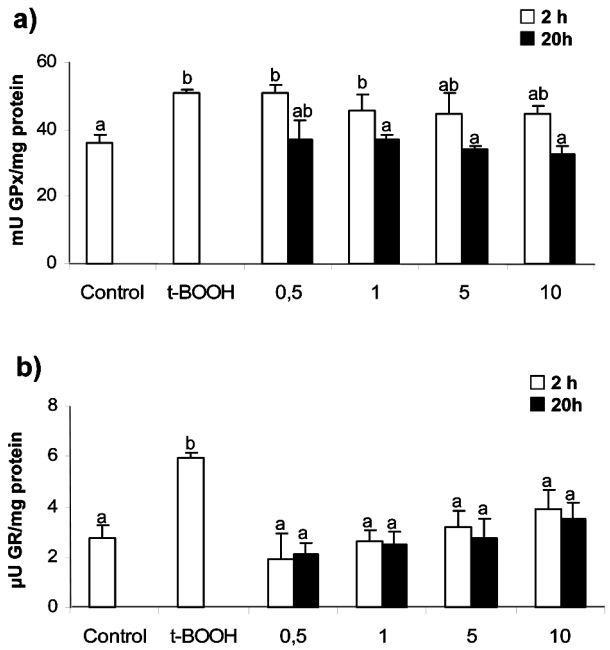

Figure 6. Experiment B: Protective effect of DCM against oxidative stress on the activity of GPx and GR evaluated in cultures of HepG2. HepG2 cells were treated with the noted µM concentrations of DCM for 2 or 20 h, then the cultures were washed and 200 µM *t*-BOOH was added for 3 h to all cultures except controls. Results from 2 and 20 h were pooled in a single bar both for control and *t*-BOOH data. Different letters indicate statistically significant differences ($p < 0.05$) among different groups. Values are means ± SD of 4–5 different samples per condition.

weight substances, such as melanoidins, are thought to be the major antioxidative compounds resulting from MRP formation [1].

Biological activities of melanoidins are similar to those reported for other natural antioxidants such as flavonoids and include prevention of LDL oxidation [40], scavenging of active oxygen [41] and hydroxyl, superoxide and peroxy radicals [22, 23] and inhibition of tumour cell growth [14]. Since the liver is not only the main target for dietary antioxidants once absorbed from the gastrointestinal tract but is the major place for xenobiotic metabolism, studies dealing with the effect of antioxidant dietary compounds at a physiological level in liver of live animals and at a cellular level in cultured cells from liver origin should be prioritary. In this line, Somoza *et al.* [42] reported an increase in the hepatic activity of chemopreventive enzymes in rats fed a 4.5% coffee beverage for 15 days. In any case, research on the antioxidant/protective effects of food melanoidins in live cultured cells from liver origin has been scarce [25].

Although MRPs may have potent antioxidant effects *in vitro* and *in vivo*, both in cell culture and live animals, elevated doses of these dietary compounds can also be toxic and mutagenic in cell culture systems and excess consumption by mammals could cause adverse metabolic reactions [39]. In fact, some of the genotoxic and redox responses to MRPs by cells may reflect prooxidant rather than antioxidant activity of these compounds. Therefore, before aiming for the protective effect of the tested antioxidant it is necessary to ensure that no direct damage is caused to the cell by the compound. Thus, in experiment A, cell toxicity and cellular redox integrity were determined in cells treated for short (4 h) and long (24 h) terms with different concentrations of DCM in the µg/mL range. No damage in cell integrity was observed after a treatment with DCM concentrations up to 100 µg/mL for 24 h. Since long term treatments are crucial when a biological effect of dietary components has to be tested, it can be assumed that the range of concentrations finally selected (0.5–10 µg/mL) can be safely used for up to 20 h in order to study the potential protective effect of dietary antioxidants against a condition of oxidative stress. On the other hand, the concentration range is realistic to evaluate the effect at the physiological level since a cup of espresso coffee provides between 6.25 and 18.75 mg/mL of melanoidin. The complete inhibition of cytotoxicity induced by *t*-BOOH when human hepatoma HepG2 cells were pretreated with DCM for 2 or 20 h, indicates that integrity of the DCM-treated cells was fully protected against the oxidative insult.

Reduced GSH is the main nonenzymatic antioxidant defence within the cell and plays an important role in protection against oxidative stress, as a substrate in GPx-catalysed detoxification of organic peroxides, by reacting with free radicals and by repairing free-radical-induced damage through electron-transfer reactions [43, 44]. It is usually assumed that GSH depletion reflects intracellular oxidation, whereas an increase in GSH concentration could be expected to prepare the cell against a potential oxidative insult [28, 31, 43, 44]. A long-term treatment (24 h) with DCM evoked a decrease in GSH levels but did not affect the GSH response to *t*-BOOH after a 20 h pretreatment, which was similar to that of 2 h. Since no direct effect of melanoidins on GSH synthesis nor interaction with thiol groups have been reported to date, no plausible explanation for the reduced GSH concentration after 24 h with DCM can be offered. In our experimental conditions, treatment of HepG2 cells with 200 µM *t*-BOOH induced a remarkable decrease in the concentration of reduced GSH which was partly prevented by pretreatment with all four doses of DCM for 2 or 20 h. Maintaining GSH concentration above a critical threshold while facing a stressful situation represents an advantage for cell survival. Other reduced soluble thiols including cysteine, gamma-glutamyl cysteine and homocysteine, are able to react with OPT and, although their relevance as compared to the reducing effect of GSH might be relatively low, some interference with the results should not be ruled out [27, 28, 31].

An important step in the degradation of cell membranes is the reaction of ROS with the double bonds of PUFAs to

yield lipid hydroperoxides. On breakdown of such hydroperoxides a great variety of aldehydes can be formed [45]. MDA, a three-carbon compound formed by scission of peroxidized PUFAs, mainly arachidonic acid, is one of the main products of lipid peroxidation [46]. Since MDA has been found elevated in various diseases thought to be related to free radical damage, it has been widely used as an index of lipoperoxidation in biological and medical sciences [47]. We have established a new method of evaluation of MDA in cultures of human hepatoma cells that is sensitive enough to detect a significant increase in MDA concentration in response to an oxidative stress induced by *t*-BOOH [33]. By using this method we have found that the *t*-BOOH-induced increase of MDA was completely avoided when cells were pretreated for 2 or 20 h with 5 – 10 μg/mL of DCM. Other than our results, determination of MDA levels in cell culture conditions is extremely scant in the literature [48]. This protection by a MRP against an induced lipid peroxidation in a cell culture is in line with previous studies that showed a similar effect by other melanoidins in primary cultures of hepatocytes [25] as well as by tea catechins [49, 50], beta carotene or lutein [49] and quercetin [31] in the same cell line, human hepatoma HepG2.

The mechanism by which melanoidins scavenge oxygen free radicals is still unclear. It has been proposed that these products contain at least one hydroxyl group, which might act as hydrogen donor and therefore have the ability to scavenge free radicals [22, 23]. Some authors have found that the high-molecular-weight fractions were more effective as antioxidants than all other fractions due not only to the direct scavenging ability of radicals but also to their stronger metal-chelating capability [21]. Direct evaluation of ROS yields a very good indication of the oxidative damage to living cells [35]. Based upon the fact that nonfluorescent 2′,7′-DCFH crosses cell membranes and is oxidized by intracellular ROS to highly fluorescent DCF [35, 51], the intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress in cells [27, 28, 31, 35]. A prooxidant such as *t*-BOOH can directly oxidize DCFH to fluorescent DCF, and it can also decompose to peroxyl radicals and generate lipid peroxides and ROS, thus increasing fluorescence. Contrary to previous reports with other dietary antioxidants [31, 52], the *t*-BOOH-induced increased ROS generation was not prevented in cultured cells pretreated for 2 or 20 h with DCM. This phenomenon is consistent with the partial recovery of the levels of reduced GSH and may be explained by an increased consumption of GSH in the enzymatic and nonenzymatic quenching of ROS generated by *t*-BOOH. These data suggest that high levels of ROS generated during the stress period are being more efficiently quenched in cells pretreated with DCM resulting in a reduced cell damage and lipid peroxidation.

In the defence against oxidative stress, the cellular antioxidant enzyme system plays a crucial role and changes in the activity of antioxidant enzymes can be considered as biomarkers of the antioxidant response [27, 28, 31, 53, 54]. GPx catalyses GSH oxidation to GSSG at the expense of H_2O_2 or other peroxides [54] and GR recycles oxidized GSH back to reduced GSH [37, 55], therefore, their activities are essential for the intracellular quenching of cell-damaging peroxide species and the effective recovery of the steady-state concentration of reduced GSH. The significant increase in the activity of GPx and GR observed after a 3 h treatment with 200 μM *t*-BOOH, clearly indicates a positive response of the cell defence system to face an oxidative insult [27, 31]. The cell enhances its enzyme defences in order to face the increasing generation of ROS such as peroxides induced by the potent prooxidant *t*-BOOH. Other dietary antioxidants have been tested by other authors and significant changes in the enzyme activity of the antioxidant enzymes have been observed only at very high doses [55, 56]. No previous cell culture study has shown a specific effect of a coffee melanoidin on the antioxidant enzymes response to an oxidative insult, but, in similar experimental conditions, we have shown that concentrations in the micromolar range of the commun flavonoid quercetin [31] and the olive oil phenolic hydroxytyrosol [52] protect cell damage by preventing the increased activity of GPx and GR induced by *t*-BOOH. In line with those results, in the present study we show, for the first time, that a long-term treatment of human hepatoma cells with μM concentrations of DCM prevents the increase in the activity of GPx and GR induced by oxidative stress. Indeed, a similar effect in GR was achieved after a short-term treatment with DCM. The results indicate that at the end of an induced stress period the antioxidant defence system of cells that had been pretreated with DCM has more efficiently returned to a steady-state activity diminishing, therefore, cell damage and enabling the cell to cope in better conditions with further oxidative insults.

In summary, our results support previous data on the antioxidant effect of synthetic melanoidins *in vitro* and extend the protective effect reported for other dietary compounds to food melanoidins contained in a highly consumed beverage, coffee. This study demonstrates that a coffee melanoidin has the ability to protect human hepatoma HepG2 cells against an oxidative insult by modulating reduced GSH concentration, MDA production and antioxidant enzymes activity. Results give more insight on the potential biological activity of food melanoidins and MRPs in general, showing that concentrations of DCM within a physiological range are able to evoke a response in cellular models. However, there are still open questions concerning their bioavailability and the mechanism by which melanoidins exert their protective effect.

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