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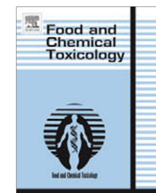

A diet rich in cocoa attenuates *N*-nitrosodiethylamine-induced liver injury in rats

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The effects of cocoa feeding against *N*-nitrosodiethylamine (DEN)-induced liver injury were studied in rats. Animals were divided into five groups. Groups 1 and 2 were fed with standard and cocoa-diet, respectively. Groups 3 and 4 were injected with DEN at 2 and 4 weeks, and fed with standard and cocoa-diet, respectively. Group 5 was treated with DEN, received the standard diet for 4 weeks and then it was replaced by the cocoa-diet. DEN-induced hepatic damage caused a significant increase in damage markers, as well as a decrease in the hepatic glutathione, diminished levels of p-ERK and enhanced protein carbonyl content, caspase-3 activity and values of p-AKT and p-JNK. The cocoa-rich diet prevented the reduction of hepatic glutathione concentration and catalase and GPx activities in DEN-injected rats, as well as diminished protein carbonyl content, caspase-3 activity, p-AKT and p-JNK levels, and increased GST activity. However, cocoa administration did not abrogate the DEN-induced body weight loss and the increased levels of hepatic-specific enzymes and LDH. These results suggested that cocoa-rich diet attenuates the DEN-induced liver injury.

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1. Introduction

Chronic liver damage is a worldwide common pathology characterized by inflammation and fibrosis that can lead to chronic hepatitis, cirrhosis and cancer (Köhle et al., 2008; Tessitore and Bollito, 2006; Thorgeirsson and Grisham, 2002). It has been well established that oxidative stress plays a causative role at the initiation, promotion and progression of hepatic diseases (Ramos, 2008; Vitaglione et al., 2004) and that the liver is the main target for several toxic agents that can provoke free radical-mediated apoptosis (Jaeschke et al., 2002).

Cocoa and its derived products are widely consumed in Europe and the United States (Vinson et al., 2006) and they have increasingly attracted researchers, food manufacturers, as well as consumers due to their biological properties, which have been mostly related to its phenolic compounds (Lamuela-Raventós et al., 2005). Cocoa flavonoids can influence several important biological functions by their free radical scavenging ability or through the regulation of signal transduction pathways to stimulate apoptosis and/or to inhibit inflammation and proliferation in different human cancer cell lines (Lamuela-Raventós et al., 2005; Martin et al., 2008, 2009; Ramos, 2007, 2008). Therefore, cocoa phenolic compounds have been suggested as potential protective and even therapeutic agents to reduce liver damage (Ramos, 2008; Vitaglione et al., 2004).

The modulation of Phase I [glutathione reductase (GR), glutathione peroxidase (GPx) and catalase (CAT)] and Phase II [glutathione-*S*-transferase (GST)] enzymes, and gluthatione (GSH) levels plays a primary role in the balance of the redox status through the reduction of reactive oxygen spices (ROS) and peroxides produced in the organism, as well as in the detoxification of xenobiotics (Ramiro-Puig et al., 2007; Ramos, 2008). Cocoa has been shown to enhance the activity of Phase I enzymes in hepatic and thymocyte cells, respectively (Martin et al., 2009; Ramiro-Puig et al., 2007), and it may also induce cell survival and proliferation, as well as protective effects, through the modulation of different transduction pathways, such as AKT and MAPKs (Martin et al., 2009; Ramiro-Puig et al., 2009). Additionally, upregulation of Phases I and II enzymes mediated by MAPKs and/or AKT has been reported for different polyphenols (Masella et al., 2005; Na and Surh, 2008). In this regard, induction of GPx and GR by cocoa has been shown to occur via ERK in hepatic cells (Martin et al., 2009). Moreover, GST expression has been implicated in the regulation of cell proliferation and protection from apoptosis through JNK-mediated mechanisms (Holley et al., 2007; Mates et al., 2008).

N-nitrosodiethylamine (DEN) is a potent hepatotoxic, carcinogen and mutagen (Chuang et al., 2000; Köhle et al., 2008; Sreepriya and Bali, 2006; Tessitore and Bollito, 2006). Human exposure could

Abbreviations: AKT/PKB, protein kinase B; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; DEN, *N*-nitrosodiethylamine; ERK, extracellular regulated kinase; FBS, fetal bovine serum; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, gluthatione; GST, glutathione-*S*-transferase; JNK, *c-jun* amino-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; OPT, *o*-phthalaldehyde.

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occur through the diet (meat, whiskey, etc.) (Hecht, 1997; Sen et al., 1980), in certain occupational settings, smoking or through the use of cosmetics, pharmaceutical products and agricultural chemicals (Hecht, 1997). DEN has been extensively used as an initiating carcinogen in experimental animal models (Chuang et al., 2000; Köhle et al., 2008; Ramakrishnan et al., 2006; Sivaramakrishnan et al., 2008; Sreepriya and Bali, 2005, 2006; Sundaresan and Subramanian, 2008; Tessitore and Bollito, 2006) and induces hepatic necrosis through metabolic activation by CYP2E1 in experimental animals (Kang et al., 2007). Activation of DEN, which takes place mainly in liver microsomes (Kang et al., 2007), has been shown to stimulate Kupfer cells leading to generate high levels of ROS, capable of damaging liver cells and participating in the induction of hepatocarcinogenesis (Kang et al., 2007). It has been shown that a cocoa bean preparation protects against different types of cancers (Yamagishi et al., 2002, 2003), as well as against a number of hepatotoxic agents including nitrosamines (Amin et al., 2004; Yamagishi et al., 2000). However, the mechanisms by which cocoa elicits such hepatoprotective and chemopreventive effects related to antioxidant defences, as well as survival/proliferation pathways, remain poorly understood.

Thus, the aim of this study was to evaluate the protective effect of a cocoa-rich diet against DEN-induced liver toxicity. Here, we report that cocoa partly attenuates DEN-induced liver injury in rats via induction of antioxidant defence (GSH, CAT, GPx and GST) and modulation of signals related to cell death (caspase-3 and JNK) and survival/proliferation (AKT).

2. Materials and methods

2.1. Materials and chemical

N-nitrosodiethylamine (DEN) was purchased from Sigma Chemical (Madrid, Spain). Anti-AKT and antiphospho-Ser473-AKT (p-AKT), anti-ERK1/2 and antiphospho-ERK1/2 (p-ERKs) recognizing ERK1/2 phosphorylated Thr202/Thy204, anti-JNK1/2 and antiphospho-JNK1/2 (p-JNKs) recognizing JNK1/2 phosphorylated Thr183/Tyr185 and anti-β-actin were obtained from Cell Signaling Technology (9271, 9272, 9101, 9102, 9251, 9252, and 4697, respectively; Izasa, Madrid, Spain). Caspase-3 substrate (Ac-DEVD-AMC) was purchased from Pharmingen (San Diego, CA). Materials and chemicals for electrophoresis and the Bradford reagent were from BioRad (BioRad Laboratories S.A., Madrid, Spain).

2.2. Cocoa

Natural Forastero cocoa powder (Nutrexpa, Barcelona, Spain) was used for this study. It contains epicatechin (383.5 mg/100 g), catechin (116 mg/100 g) and procyanidins (254.5 mg/100 g) and non-flavonoid compounds such as theobromine. A detailed description of this cocoa is given elsewhere (Martin et al., 2008).

2.3. Animal treatments

Forty five male Sprague–Dawley rats (6 weeks old) were obtained from the School of Medicine, Universidad Autónoma (Madrid, Spain). Animals were placed individually in stainless steel wire-bottomed metabolic cages housed in a room under controlled conditions (19–23 °C, 50–60% humidity and 12 h light/darkness cycles).

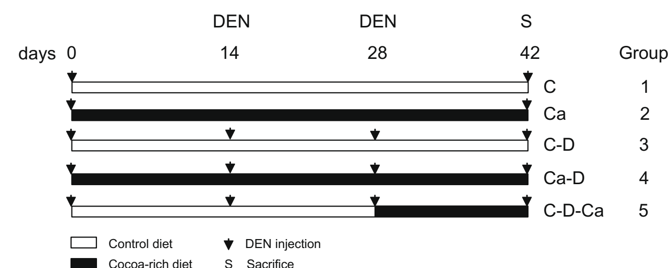

Fig. 1. Schematic representation of the experimental design used for the study.

Diets were prepared from a Fibre Free AIN-93 M Purified Rodent Diet (Panlab S.L., Barcelona, Spain) providing all nutrients required by adult rats. Rats were randomly assorted into five different experimental groups (8–10 animals per group) (Fig. 1) and were provided with food and water *ad libitum*. Initially, three groups received a standard diet (C, Groups 1, 3 and 5) and two groups were fed with cocoa-rich diet (Ca, Groups 2 and 4), containing the basal diet supplemented with 16% powdered cocoa (providing 54.4 g of dietary fibre per kg of diet), added as a source of polyphenols. In line with our previous research, this powdered supplement was formulated to provide 7.5 g of polyphenols per kg of diet, since this percentage of cocoa supplementation improved the antioxidant defence system, the lipid profile and reduced the lipid peroxidation. (Lecumberri et al., 2007). The composition of the diets is given in Table 1 and cocoa powder contains 26% proteins and 12% lipids.

To induce hepatic damage, three groups of rats (Groups 3–5) were injected intraperitoneally with 200 mg/kg DEN diluted in saline on days 14 and 28. Rats in the non-DEN-treated groups were injected with saline the same day. In addition, on day 28, Group 5 was changed from control diet to cocoa-diet after the second DEN injection (Fig. 1) to test the potential therapeutic activity of the cocoa-rich diet.

Animal weight and food intake were monitored daily for 6 weeks. Blood was harvested from the trunk after decapitation, and serum was separated by centrifugation at 1000*g*, 10 min, 4 °C for further biochemical analysis. Livers were collected, weighted and frozen in liquid N_2 and stored at −80 °C.

Animals were treated according to the Institutional Care Instructions (Bioethical Commission from Consejo Superior de Investigaciones Cientificas).

2.4. Analysis of hepatic enzymes (AST, ALT, ALP)

As markers for liver function in serum, the activity of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) [expressed as Units (U)/L serum] were analysed. Serum AST was measured spectrophotometrically at 340 nm in the presence of α-ketoglutarate, aspartate, NADH and malate dehydrogenase, following the method of Rej and Horder (1984). Serum ALT was assayed at 340 nm in the presence of α-ketoglutarate, pyruvate, NADH and lactate dehydrogenase as described by Horder and Rej (1984). Serum ALP was analysed spectrophotometrically at 405 nm using the formation of *p*-nitrophenol from *p*-nitrophenylphosphate as a substrate (Principato et al., 1985). Serum protein concentration was measured by the Bradford reagent.

2.5. Determination of GSH

The content of GSH was quantitated by the fluorometric assay of Hissin and Hilf (1976). The method takes advantage of the reaction of GSH with *o*-phthalaldehyde (OPT) at pH 8.0, which generates fluorescence.

Livers were homogenized (1:20 w/v) in 50 mM phosphate buffer pH 7.0, proteins precipitated with 5% trichloroacetic acid and then centrifuged for 30 min at 10,000*g*. Following, 50 μL of the clear supernatant were transferred to a 96 multi-well plate for the assay. Fluorescence was measured at an excitation wavelength of 340 nm and emission wavelength of 460 nm. The results were interpolated in a glutathione standard curve (5 ng-1 μg) and expressed as nmol GSH per milligram of protein, which was determined by the Bradford reagent. The precise protocol has been described elsewhere (Alía et al., 2003, 2006).

2.6. Determination of GPx, GR, CAT and GST activities

The activity of antioxidant enzymes (GPx, GR and catalase) was determined in liver homogenates. Livers (0.3 g) were homogenized (1:5 w/v) in 0.25 M Tris, 0.2 M sucrose and 5 mM 1,4-dithiothreitol (DTT) buffer pH 7.4 and centrifuged at 3000*g* for 15 min. Determination of GPx activity is based on the oxidation of GSH by

Table 1
Composition of the experimental control (C) and cocoa-rich (Ca) diets (g/kg dry weight).

	C	Ca
Casein	140	140
Dextrose	155	155
Sucrose	100	100
Fat	40	40
t-BHQ[a]	0.008	0.008
Mineral mix.	35	35
Vitamin mix.	10	10
L-Cys	1.8	1.8
Cholin bitartrate	2.5	2.5
Cellulose	100	45.6
Starch	415.7	310.1
Cocoa powder	–	160

[a] *tert*-Butylhydroquinone.

GPx, using t-BOOH as a substrate, coupled to the disappearance of NADPH by GR (Gunzler et al., 1974), expressing the results as mU/mg protein. GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione (Goldberg and Spooner, 1987). The GR activity was expressed as μU/mg protein. Catalase (CAT) activity was determined by following the decomposition of H_2O_2 measured as a decrease in absorbance at 240 nm (Aebi, 1987) and expressed as UI/mg protein. The methods have been previously described (Alía et al., 2003, 2006).

Analysis of GST was carried out in liver and serum by the Biovision Commercial kit GST fluorometric activity assay (Biovision, Mountain View, CA) and the activities were expressed as U/mg protein and mU/mg protein, respectively. Protein was measured by the Bradford reagent.

2.7. Protein carbonyl determination in liver

Protein oxidation of liver homogenates was measured as carbonyl groups content according to the method of Richert et al. (2002). Absorbance was measured at 360 nm and carbonyl content was expressed as nmol/mg protein using an extinction coefficient of 22000 nmol L^{-1} cm^{-1}. Protein in liver homogenates was determined by the Bradford reagent.

2.8. Lactate dehydrogenase (LDH) leakage assay

Livers were homogenized (1:5 w/v) in 50 mM phosphate buffer (PBS) pH 7.5, centrifuged for 30 min at 1000*g* and the supernatants collected. LDH was spectrophotometrically assayed in the samples by measuring the disappearance of NADH at 340 nm, as previously described (Alía et al., 2006; Bergmeyer and Bernt, 1974). The reaction mixture contained 5 mM pyruvate, 0.35 mM NADH, 84 mM Tris and 50 μg of protein per condition. Enzyme activity was calculated by using an extinction coefficient of 6.22 mM^{-1} cm^{-1} and expressed in units using mg^{-1} protein. One unit of the enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol NADH min^{-1}.

2.9. Liver caspase-3 activity

Activation of caspase-3 was determined as previously described (Granado-Serrano et al., 2007). Briefly, livers were lysed (1:5 w/v) in a buffer containing 5 mM Tris (pH 8), 20 mM EDTA, and 0.5% Triton X-100. The reaction mixture contained 20 mM HEPES (pH 7), 10% glycerol, 2 mM DTT, 50 μg of protein per condition and 20 μM Ac-DEVDAMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) as substrate. Enzymatic activity was determined by measuring fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 440 nm (Bio-Tek, Winooski, VT, USA).

2.10. Preparation of cell lysates for Western blotting

Samples of frozen liver were homogenized 1:10 (w:v) in extraction buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM EDTA, 1% Nonidet P-40, 2 mM Na_3VO_4, 5 μg/mL leupeptin, 20 μg/mL aprotinin, 2 mM benzamidin and 2 mM phenylmethylsulphonyl fluoride (PMSF)] to detect AKT, p-AKT, ERK1/2, p-ERKs, JNK1/2 and p-JNKs (Gavete et al., 2005). Homogenates were centrifuged at 14,000*g* for 60 min and the supernatants were collected, assayed for protein concentration by using the Bradford reagent, aliquoted and stored at −80 °C until use for Western blot analyses.

2.11. Protein determination by Western Blotting

Equal amounts of protein (100 μg) were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) filters (Protein Sequencing Membrane, BioRad). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated antirabbit Ig (GE Healthcare, Madrid, Spain). Blots were developed with the ECL system (GE Healthcare, Madrid, Spain). Normalization of Western blot was ensured by β-actin and band quantification was carried out with a scanner and the Scion Image software.

2.12. Statistics

Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene. For multiple comparisons, one-way ANOVA was followed by the Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. $P < 0.05$ was considered significant. A SPSS version 15.0 program was used.

3. Results

3.1. Body and liver weights and food consumption

Final body weights and food consumption decreased after DEN administration in all groups of animals (Groups 3–5) (Table 2). Consistent with these results, body weight gains of Groups 1 and 2 were higher than those in DEN-injected groups. The lowest values were observed in Group 5 corresponding to DEN-treated animals that received the cocoa-rich diet at day 28, after being fed with standard diet (Table 2). Therefore, the food efficiency diminished in Groups 3–5 when compared to DEN-untreated animals (Groups 1 and 2), and the smallest alimentary efficiencies were observed in DEN-rats receiving the cocoa-rich diet (Groups 4 and 5).

Table 2
Body weight data, food intake and liver weight of rats in control and DEN groups fed with standard (C) and cocoa-rich (Ca) diets.[A]

	Control groups		DEN-groups		
	1 (C)	2 (Ca)	3 (C)	4 (Ca)	5 (C–Ca)
Initial body weight (g)	95.00 ± 4.69[a]	92.57 ± 3.41[a]	102.75 ± 8.87[a]	104.00 ± 7.77[a]	105.88 ± 5.26[a]
Body weight on day 14 (g)	135.17 ± 3.81[a]	134.00 ± 7.83[a]	135.00 ± 5.58[a]	134.33 ± 6.68[a]	137.75 ± 7.89[a]
Final body weight (g)	205.83 ± 10.21[a]	202.14 ± 6.36[a]	183.17 ± 8.18[b]	175.71 ± 12.09[bc]	161.29 ± 10.81[c]
Body weight gain (g in 42d)	110.83 ± 7.92[a]	110.57 ± 4.58[a]	81.20 ± 8.32[b]	71.71 ± 16.98[bc]	57.67 ± 17.15[c]
Food intake (g in 42 d)	574.58 ± 3.66[a]	574.40 ± 6.31[a]	529.58 ± 25.66[b]	534.91 ± 10.78[b]	531.91 ± 16.53[b]
Food efficiency (body weight gain/food intake)	0.19 ± 0.02[a]	0.19 ± 0.01[a]	0.16 ± 0.02[b]	0.13 ± 0.02[c]	0.11 ± 0.03[c]
Liver weight (g)	4.32 ± 0.28[a]	4.42 ± 0.28[a]	4.32 ± 0.17[a]	4.20 ± 0.22[ab]	3.79 ± 0.14[b]
Liver-to-body weight ratio	0.021 ± 0.002[a]	0.022 ± 0.002[a]	0.022 ± 0.001[a]	0.024 ± 0.002[a]	0.023 ± 0.002[a]

[A] Data represent the means ± SD. Means in a row without a common letter differ, $P < 0.05$.

Table 3
Serum levels of total protein and activity of hepatic enzymes in control and DEN groups fed with standard (C) and cocoa-rich (Ca) diets.[A]

	Control groups		DEN-groups		
	1 (C)	2 (Ca)	3 (C)	4 (Ca)	5 (C-Ca)
Total protein (mg/mL)	112.09 ± 11.61[a]	119.09 ± 12.87[a]	80.16 ± 11.65[b]	80.96 ± 10.43[b]	76.35 ± 16.55[b]
ALT (U/L)	51.50 ± 10.67[a]	59.29 ± 12.47[ab]	75.50 ± 12.33[bc]	85.00 ± 6.73[cd]	86.75 ± 8.54[d]
AST (U/L)	284.80 ± 41.84[a]	278.17 ± 32.18[a]	265.17 ± 28.99[a]	479.67 ± 83.46[b]	435.33 ± 67.33[b]
ALP (U/L)	202.00 ± 20.82[a]	221.00 ± 25.91[a]	338.33 ± 33.43[b]	315.00 ± 47.93[b]	342.20 ± 54.67[b]
GST (mU/mg protein)	0.58 ± 0.07[a]	0.46 ± 0.08[a]	0.56 ± 0.09[ab]	0.71 ± 0.07[b]	0.49 ± 0.09[a]

[A] Data represent the means ± SD. Means in a row without a common letter differ, $P < 0.05$.

Liver weights of Group 5 were diminished when compared to all other groups of animals. However, no significant differences in liver-to-body weight ratio were observed among the experimental groups (Table 2).

3.2. Levels of markers of liver function in serum

As shown in Table 3, the serum total protein levels were reduced in DEN-treated rats, and were not normalized by the cocoa supplementation; on the contrary, Group 5, in which diet was changed from standard to cocoa-rich one, showed the lowest values (Table 3).

The serum activity of ALT exhibited an elevation in DEN-treated groups and remained increased in animals receiving cocoa-diet in comparison with untreated rats (Table 3). Addition of cocoa to DEN-injected animals (Groups 4 and 5) showed an enhanced activity of AST when compared to untreated (Groups 1 and 2) and DEN-treated (Group 3) rats. Similarly, ALP activity was increased because of the administration of DEN in all experimental groups (Groups 3–5). However, only in cocoa-fed rats treated with DEN (Group 4), an increase in GST activity was observed (Table 3).

3.3. Levels of liver GSH and Phases I and II enzymes

Liver GSH concentration decreased by DEN administration in Group 3, which showed the lowest levels (Fig. 2). However, liver GSH content was elevated by cocoa in control and DEN-treated rats (Groups 2 and 4, Fig. 2), whereas animals fed with cocoa-diet for 2 weeks (Group 5) displayed similar values to control DEN-untreated rats (Group 1).

Similar results were obtained for GPx activity, with increased values in the animals consuming cocoa for 6 weeks (Groups 2 and 4), decreased GPx activity after DEN treatment in rats fed with standard diet (Group 3), and recovering of control values in Group 5. GR showed an increased activity in untreated animals fed cocoa (Group 2), while all the DEN-treated groups showed similar values to controls (Group 1) (Fig. 2). Similarly to GPx, catalase activity decreased in animals fed with standard diet and treated with DEN (Group 3), whereas the other groups of rats displayed similar levels to controls (Fig. 2). GST activity in liver remained unaltered in all animals except in Group 4, where an increase in its activity was detected (Fig. 2). Therefore, cocoa induces the antioxidant defence in the liver of the control and DEN-injected rats.

3.4. Protein carbonyl content and LDH levels and caspase-3 activity in liver

Levels of protein carbonyl content were increased in Group 3 due to DEN administration when compared to no-injected animals (Fig. 3). However, cocoa-fed rats treated with DEN (Groups 4 and 5) showed decreased protein carbonyl levels when compared to

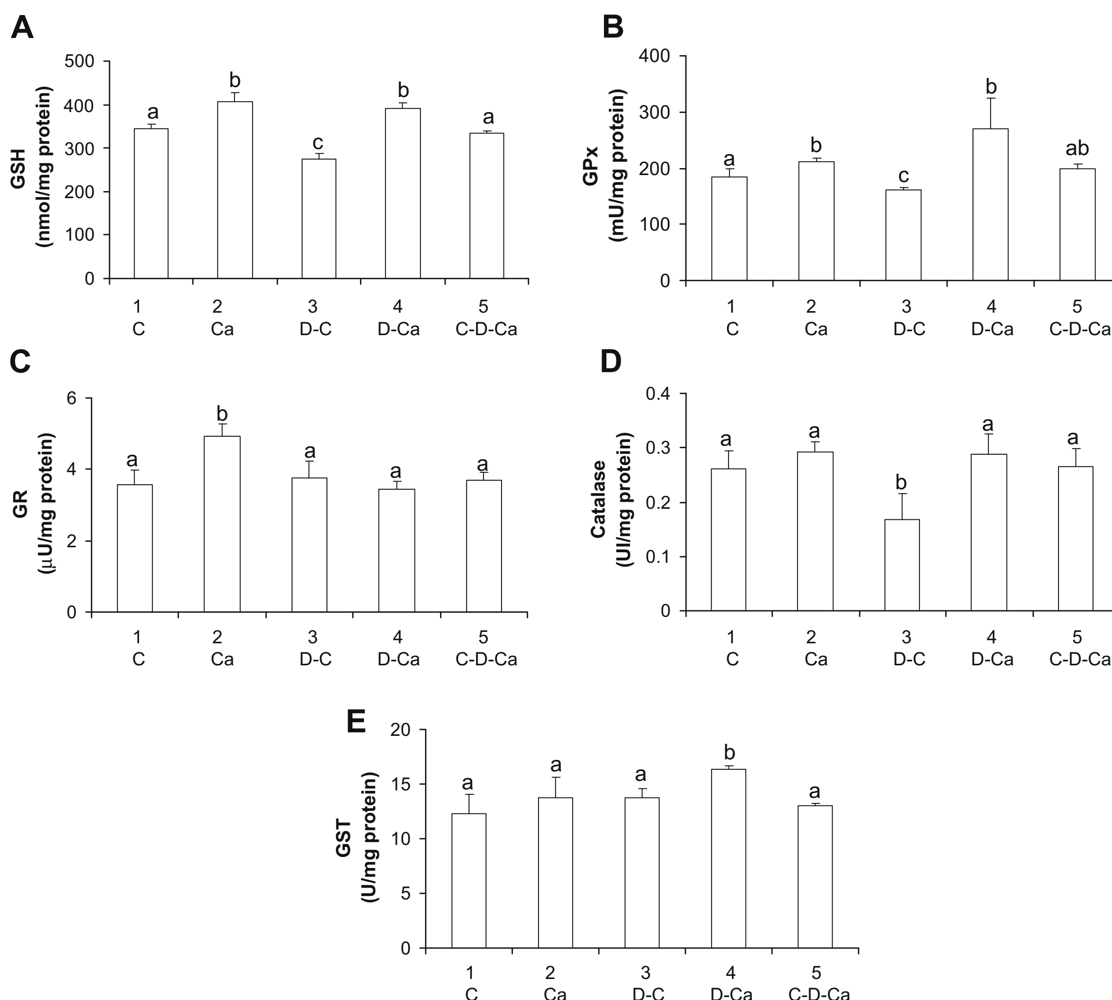

Fig. 2. Effect of cocoa on: (A) glutathione levels and activity of (B) GPx, (C) GR, (D) CAT and (E) GST in liver from control and DEN-groups fed with standard (C) and cocoa-rich (Ca) diets. Data represent the means ± SD (n = 8–10). Means without a common letter differ, $P < 0.05$.

Group 3, values were reverted to control levels in Group 4 (cocoa-fed during 4 weeks) or partly diminished in Group 5 (cocoa-fed for 2 weeks).

Lactate dehydrogenase levels were increased by DEN administration in all groups (Groups 3–5), pointing at a DEN-induced liver injury that was not prevented by the cocoa-rich diet (Fig. 3). DEN also induced caspase-3 activity (Group 3), but cocoa administration was capable of returning caspase-3 activity to control levels in animals consuming the cocoa-rich diet throughout the experiment (Group 4), or partly decreased the DEN-induced activation of caspase-3 in animals receiving the cocoa-rich diet only for two weeks (Group 5). Animals fed with the cocoa-rich diet (Group 2) showed LDH levels similar to control rats and decreased values of caspase-3 activity (Fig. 3). These results indicate that cocoa diminished the oxidative (carbonyl protein content) and apoptotic damages (caspase-3 activation), but not the necrotic DEN-induced liver injury (LDH activity).

3.5. Liver AKT, ERK and JNK levels

Figs. 4 and 5 illustrate that DEN-treated animals fed with the standard diet (Group 3) showed a significant increase in the levels of phosphorylated AKT and JNK proteins, whereas no changes in these two proteins were found in the other groups of animals. Phosphorylated ERK levels were increased by cocoa administration in control animals (Group 2), but they were dramatically decreased by DEN treatment in all injected rats (Groups 3–5) (Fig. 5). There was no difference in the total levels of AKT, ERKs and JNKs among the groups. Thus, cocoa prevented the activation of AKT and JNK, although it did not modify the decreased values of active ERK induced by DEN.

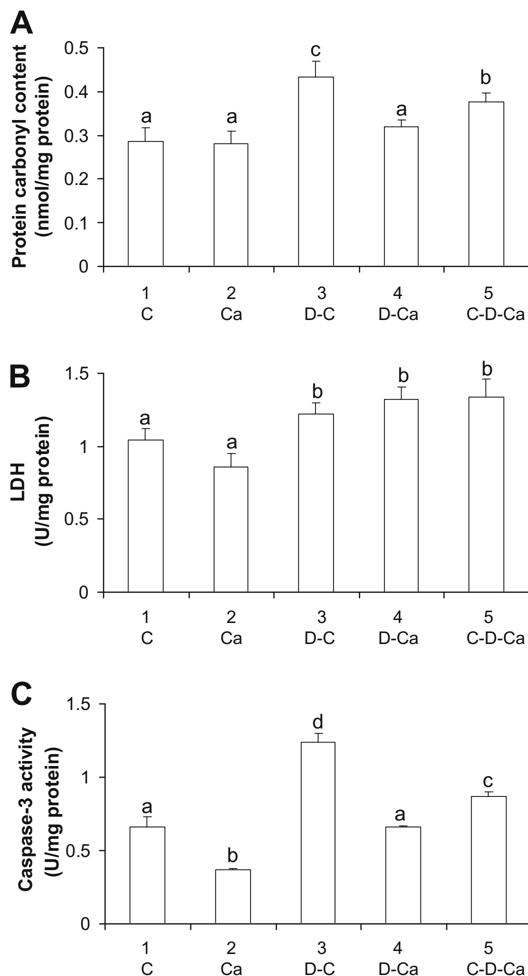

Fig. 3. Liver (A) protein carbonyl (B) LDH and (C) caspase-3 levels in control and DEN-groups fed with standard (C) and cocoa-rich (Ca) diets. Data represent the means ± SD (n = 6–8). Means without a common letter differ, $P < 0.05$.

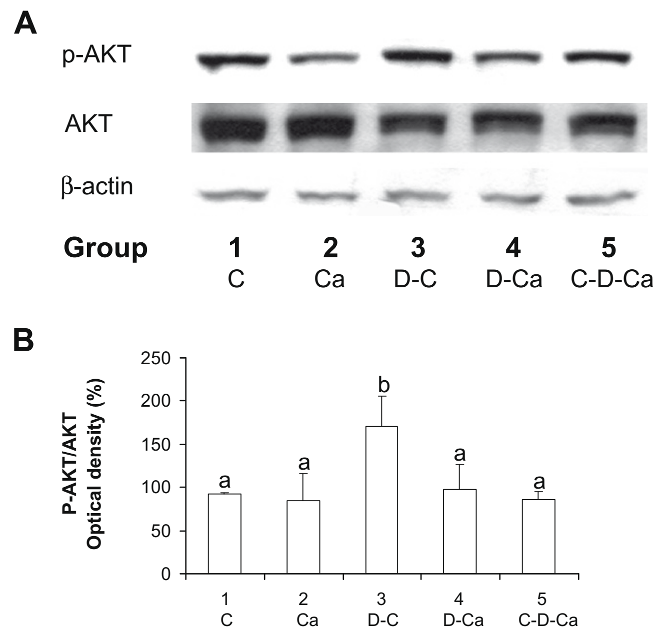

Fig. 4. Effect of cocoa on levels of phosphorylated AKT (Ser473) and total AKT in controls and DEN-treated rats. (A) Bands of a representative experiment. (B) Percentage values of the p-AKT/AKT ratio relative to the control condition (means ± SD, n = 6). Normalization of Western blots was ensured by β-actin. Means without a common letter differ, $P < 0.05$.

4. Discussion

DEN toxicity is primarily associated to an excessive production of free radicals in the liver. As a consequence, reactive electrophilic intermediates are formed, which overwhelms the antioxidant defences and ultimately proceeds to oxidative stress paving way to liver damage (Kang et al., 2007). DEN induces a postnecrotic hepatocellular proliferation that contributes to enhance the number of initiated cells (Cascales, 2001), and it is accepted as a model to study the relations among liver necrosis, cancer initiation and replication (Cascales, 2001; Chuang et al., 2000; Köhle et al., 2008; Ramakrishnan et al., 2006; Sivaramakrishnan et al., 2008; Sreepriya and Bali, 2005, 2006; Sundaresan and Subramanian, 2008; Tessitore and Bollito, 2006).

Cocoa and its derived products have been demonstrated to contain important antioxidant polyphenols that inhibit different tumoral processes and exhibit antioxidant and anti-inflammatory properties (Amin et al., 2004; Lamuela-Raventós et al., 2005; Martin et al., 2008, 2009; Yamagishi et al., 2000, 2002, 2003). In this study, we show that a cocoa-rich diet partly protect the liver against an oxidative-induced damage by decreasing the protein carbonyl content and modulating the activities of antioxidant enzymes and by regulating key proteins of cell signalling cascades, although cocoa-rich diet was unable to abrogate the DEN-induced enhanced values of hepatospecific enzymes and LDH. The cocoa dose was finally selected based on our own previous in vivo studies, where it is showed that it contributed to reduce the cardiovascular risk acting as an effective antioxidant (Lecumberri et al., 2007).

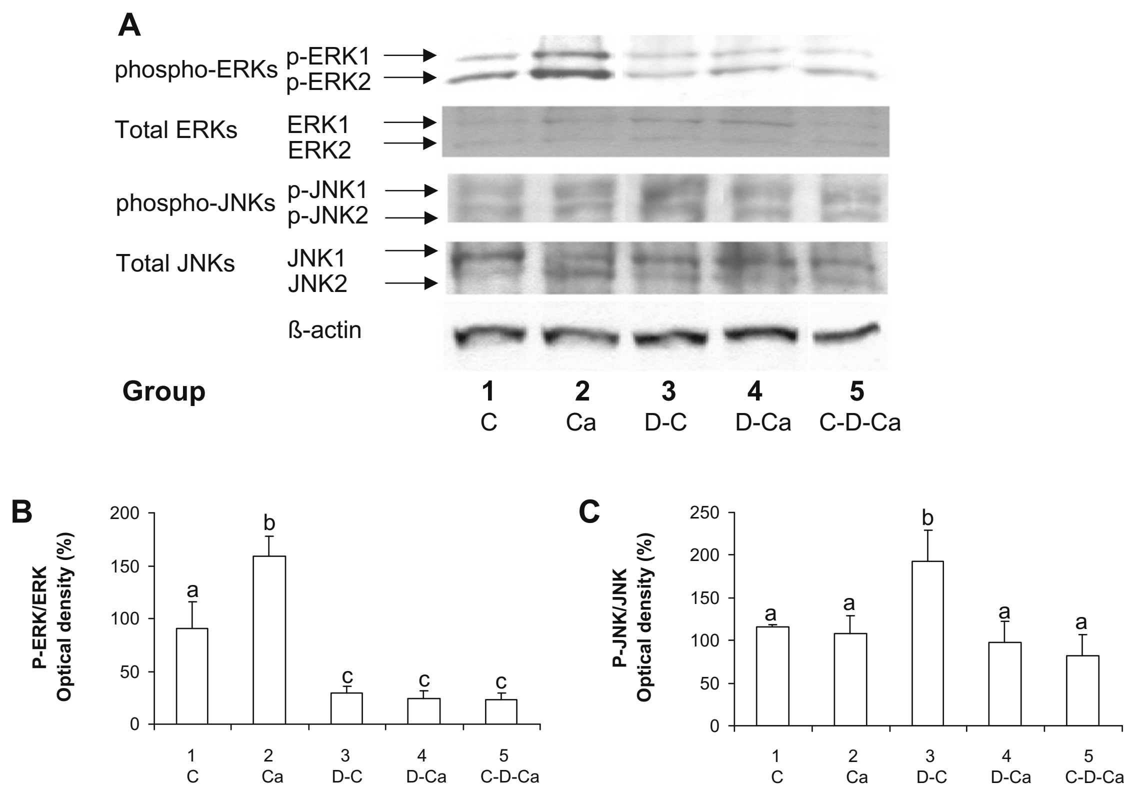

Fig. 5. Effects of cocoa on the basal levels of phosphorylated ERK1/2 (Thr202/Tyr204), total ERK1/2, phosphorylated JNK1/2 (Thr183/Tyr185) and total JNK in controls and DEN-treated animals. (A) Representative blots of both MAPKs. Percentage data of (B) p-ERK/ERK and (C) p-JNK/JNK ratios relative to controls (means ± SD, $n = 7$). The same liver homogenates were subjected to Western blot analysis using the corresponding non-phospho-specific antibodies to detect total ERK or JNK. Equal loading of Western blots was ensured by β-actin. Means without a common letter differ, $P < 0.05$.

Reduction of food intake and consequently, the reduction of body weight gain observed in DEN-treated animals, could be largely due to losses from skeletal muscle and adipose tissue as previously shown (Sreepriya and Bali, 2005), and it could be considered as an indirect indication of the declining hepatic function following exposure to DEN (Amin et al., 2004; Ramakrishnan et al., 2006; Sivaramakrishnan et al., 2008; Sreepriya and Bali, 2005). In addition, assessment of the liver/body weights ratio was used to investigate potential changes in the liver size, but no differences were found (Ramakrishnan et al., 2006; Sreepriya and Bali, 2005; Yamagishi et al., 2003).

Low levels of albumin have been reported in the serum of patients and animals with early hepatocellular cancer (Sreepriya and Bali, 2005; Yoshida et al., 2005). Contrary to other antioxidants such as curcumin and embelin that reverted the diminished serum proteins values induced by DEN (Sreepriya and Bali, 2005), our results indicate that cocoa did not exhibit its protective effects during DEN-induced liver damage by enhancing the total protein levels.

GSH is an important non-enzymatic antioxidant defence required to maintain the normal redox state of cells and to counteract deleterious effects of oxidative stress. GSH depletion ultimately promotes oxidative stress, with a cascade of effects thereby affecting functional and structural integrity of cell and organelle membrane (Masella et al., 2005). Our results showed that the significant decrease in GSH levels in animals exposed to DEN was restored or enhanced by cocoa feeding, which indicated the potentiality of cocoa to counteract the oxidative damage induced by DEN and to reinforce the antioxidant defence in normal conditions, as previously reported for other natural antioxidants (Ramakrishnan et al., 2006; Sivaramakrishnan et al., 2008; Sreepriya and Bali, 2006).

Chemical induction of liver damage by DEN administration is associated with changes in the oxygen radical metabolism in this organ, which were demonstrated by measuring protein carbonyl content and the activity of the antioxidant enzymes. Generation of protein, DNA and lipid oxidation products by DEN administration at the initiation stage can be prevented by natural antioxidants (Ramakrishnan et al., 2006; Sivaramakrishnan et al., 2008; Sreepriya and Bali, 2006). Thus, DEN-treated rats fed with cocoa (Groups 4 and 5) displayed lower levels of protein carbonyl content when compared with rats injected with DEN receiving regular diet (Group 3). This reveals the protective role of cocoa against the induced oxidative stress (Lecumberri et al., 2007; Martin et al., 2008; Ramiro-Puig et al., 2009).

Catalase converts H_2O_2 to H_2O and GPx catalyses the transformation of H_2O_2 to harmless byproducts. During H_2O_2 scavenging, GSH is oxidized to GSSG by GPx. The reduction of GSSG to GSH is catalysed by GR using NADPH as reducing potential. GPx and GR activities were increased and catalase activity was unchanged in control animals fed cocoa in agreement with our previous results, where the induction of both enzymatic activities by a cocoa polyphenolic extract (Martin et al., 2009) and a cocoa fibre byproduct (Lecumberri et al., 2007) was demonstrated. The decrease of GSH levels and GPx and catalase activities indicates the severity of the oxidative stress-induced during the exposure to DEN. Interestingly, cocoa counteracted the hepatic oxidative damage by preventing the reduction of these parameters provoked by DEN. In line with this, decreases in GSH levels, as well as in activities of catalase, GPx and GR, have been reported in hepatic tumours (Ramakrishnan et al., 2006; Sivaramakrishnan et al., 2008; Sreepriya and Bali, 2006).

GST catalyses the reaction of endogenous GSH with numerous electrophiles to yield less toxic conjugates that are easily eliminated (Masella et al., 2005). Although increased and decreased liver and

serum GST activities have been reported after DEN-induced damage (Amin et al., 2004; Sivaramakrishnan et al., 2008; Sreepriya and Bali, 2006; Sundaresan and Subramanian, 2008; Yadav and Bhatnagar, 2007), we found that liver and serum GST activities increased in rats fed with cocoa and injected with DEN (Group 4), suggesting that cocoa administration contributes to the liver protection against the oxidative-induced-injury. In this regard, it has been demonstrated that different natural compounds and polyphenols, as well a cocoa bean product, induce GST as one of the principal anticarcinogenic mechanisms (Amin et al., 2004; Masella et al., 2005; Sundaresan and Subramanian, 2008). Moreover, some studies point to the induction of GST as a mechanism to protect against chemically induced cancer and oxidative stress by increasing the metabolism of electrophilic intermediates and ROS (Masella et al., 2005).

Enhancement of liver LDH activity, a sensitive marker of hepatocyte injury, indicates a nonspecific alteration in the plasma membrane integrity and permeability and/or may be due to its overproduction by tumour cells (Kamaraj et al., 2007; Manso et al., 2007; Sivaramakrishnan et al., 2008). The significant DEN-induced increase in serum hepatic marker enzymes and liver LDH activities that indicated the hepatocellular dysfunction and the severity of the liver necrotic damage (Amin et al., 2004; Kamaraj et al., 2007; Sivaramakrishnan et al., 2008), were not avoided by the administration of a cocoa-rich diet.

Activation of caspase-3, the most important enzyme responsible for apoptosis, has been considered as a sensitive method of detecting liver damage and has been associated with progressive liver fibrosis (Bantel et al., 2004). Moreover, high rates of apoptosis have been demonstrated in liver cancer (Kang et al., 2007; Schimtz et al., 2007). In addition, it should be mentioned that administration of DEN (200 mg/kg) causes fibrosis and necrosis in the liver (Tessitore and Bollito, 2006). It is noticeable that DEN-injected rats fed with standard diet (Group 3) showed levels of AST and ALT that were below those of the other DEN-treated groups. This finding could be a consequence of a reduction in the number of viable hepatocytes due to enhanced cell death in liver, as these animals also showed the highest levels of LDH and caspase-3 (Manso et al., 2007). Thus, the decreased caspase-3 activity in animals not injected with DEN and fed with cocoa (Group 2), as well as in DEN-treated rats receiving cocoa (Groups 4 and 5), suggested a potential protective effect of cocoa against cell death in normal and under oxidative conditions. Therefore, although additional studies are needed, it could be suggested that cocoa could partly protect hepatocytes against the progression of the fibrotic damage induced by DEN.

The signalling mechanisms associated with liver damage induced by oxidative stress are not completely known. In our study, the increase in p-AKT levels in DEN-treated rats fed the standard diet (Group 3) agrees with that reported in animals receiving DEN in drinking water (Parekh and Rao, 2007). These data support the important role of AKT in controlling the balance between survival and apoptosis (Ramos, 2008), which has been considered a critical factor in the aggressiveness of hepatocellular cancer (Parekh and Rao, 2007; Schimtz et al., 2007). Moreover, the value of p-AKT was restored in rats injected with DEN but fed cocoa (Groups 4 and 5) suggests that cocoa prevents the activation of this main protein related to cell survival/proliferation; this could result in a potential attenuation of the postnecrotic proliferation induced by DEN and in a reduction of the number of initiated cells (Cascales, 2001).

ERK plays a critical role in controlling the balance between cell survival and proliferation, and cell cycle progression (Schimtz et al., 2007). Although data on p-ERK values remain controversial (Ito et al., 1998; Parekh and Rao, 2007), increased p-ERK levels have recently been related to an advanced, but not early, tumour stage (Huynh et al., 2003). This finding supports our results, since decreased levels of p-ERK were found in all DEN-injected animals, a model to study the relations among necrosis and cancer initiation. Moreover, p-ERK was increased in rats fed a cocoa-rich diet indicating that cocoa components can activate cellular kinases (Ramos, 2008) in concert with previous results (Granado-Serrano et al., 2007; Martin et al., 2009; Ramiro-Puig et al., 2009). Similarly, the enhanced levels of p-ERK in cocoa-fed rats (Group 2) could be related to their increased GR and GPx activities, since MAPKs pathway has recently been implicated in the upregulation of several antioxidant enzymes activities in liver cancer cells (Martin et al., 2009).

The JNK family belongs to the MAPKs superfamily (same as ERKs) and its activation has been associated to apoptosis in liver (Czaja, 2003; Parekh and Rao, 2007). DEN administration enhanced the p-JNK/JNK ratio in animals fed with the standard diet (Group 3) and returned to control values in DEN-groups receiving cocoa (Groups 4 and 5). In line with this, a protective effect of cocoa flavonoids has been described on a model of oxidative stress in neurons, showing a down-regulation of p-JNK levels (Ramiro-Puig et al., 2009). Moreover, DEN-treated rats fed with regular diet showed the highest LDH and caspase-3 activities and p-JNK/JNK values, pointing out to an enhanced cell death in the liver of these animals, although this group also exhibited enhanced p-AKT values suggestive of a promotion of cellular survival/proliferation. At the moment we cannot explain this finding and further studies are needed to elucidate this result, but similar effects have been reported in patients with liver cancer (Kang et al., 2007; Schimtz et al., 2007).

Finally, it is important to mention that in vitro studies have attributed the protective effect of cocoa to the polyphenols, since the contribution of theobromine was considered negligible (Martin et al., 2008). Additionally, a similar protective effect has been recently described after treating cells with a cocoa phenolic extract or (−)-epicatechin (Ramiro-Puig et al., 2009). Therefore, it could be suggested that the beneficial effects of cacao in the present study might be ascribed to the polyphenolic compounds, which even may develop synergism among them, but further studies are needed.

In summary, a cocoa-rich diet protects cells against DEN-induced oxidative stress by activating the antioxidant defence system. In addition, cocoa appears to exert an antiapoptotic effect in the liver of DEN-treated animals that could be associated with the prevention of JNK and caspase-3 activation. Cocoa also prevented the DEN-induced increase of the survival/proliferation signal AKT, associated with poor prognosis in liver cancer. All these effects contribute to attenuate the liver DEN-induced oxidative damage. However, cocoa was not completely capable of avoiding the liver necrotic damage induced by the hepatotoxic as shown by the increased activities of the hepatospecific enzymes and LDH as well as decreased p-ERK values. These results provide new insights into the antioxidative mechanisms of cocoa flavonoids and point towards their antiapoptotic and modulatory effects on cell death and survival/proliferation pathways as additional mechanisms of action of these compounds. Therefore, it could be suggested that cocoa or cocoa products enriched in flavonoids, which lack of toxicity in humans, may contribute to the protection against liver oxidative stress-related diseases.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

Aebi, H., 1987. Catalase. In: Bergmeyer, H.V. (Ed.), Methods of Enzymatic Analysis. Verlag-Chemie, Weinheim, Germany, pp. 273–282.

Alia, M., Horcajo, C., Bravo, L., Goya, L., 2003. Effect of grape antioxidant dietary fiber on total antioxidant capacity and the activity of liver antioxidant enzymes in rats. Nutr. Res. 23, 1251–1267.

Alía, M., Ramos, S., Mateos, R., Granado-Serrano, A., Bravo, L., Goya, L., 2006. Quercetin protects human hepatoma cell line (HepG2) against oxidative stress induced by tertbutyl hydroperoxide. Toxicol. Appl. Pharmacol. 212, 110–118.

Amin, I., Koh, B., Asmah, R., 2004. Effect of cacao liquor on tumor marker enzymes during chemical hepatocarcinogenesis in rats. J. Med. Food. 7, 7–12.

Bantel, H., Lügering, A., Heideman, J., Volkmann, X., Poremba, C., Strassburg, C., Manns, M., Schulze-Osthoff, K., 2004. Detection of apoptotic caspase activation in sera from patients with chronic HCV infection is associated with fibrotic liver injury. Hepatology 40, 1078–1087.

Bergmeyer, H., Bernt, E., 1974. Lactic dehydrogenase. Meth. Enzym. Anal. 2 (57), 4–579.

Cascales, M., 2001. Mecanismos de hepatotoxicidad. Real Academia de Doctores, Madrid.

Czaja, M., 2003. The future of GI and liver research: Editorial perspectives III. JNK/AP-1 regulation of hepatocyte death. Am. J. Physiol. Gastrointest. Liver Physiol. 284, G875–G879.

Chuang, S., Cheng, A., Lin, J., Kuo, M., 2000. Inhibition by curcumin of diethylnitrosamine-induced hepatic hyperplasia, inflammation, cellular gene products and cell-cycle-related proteins in rats. Food Chem. Toxicol. 38, 991–995.

Gavete, L., Martin, M., Alvarez, C., Escriva, F., 2005. Maternal food restriction enhances insulin-induced GLUT-4 translocation and insulin signaling pathway in skeletal muscle from suckling rats. Endocrinology 146, 3368–3378.

Goldberg, D., Spooner, R., 1987. Glutathione reductase. In: Bergmeyer, H.V. (Ed.), Methods of Enzymatic Analysis. Verlag-Chemie, Weinheim, Germany, pp. 258–265.

Granado-Serrano, A., Martín, M., Izquierdo-Pulido, M., Goya, L., Bravo, L., Ramos, S., 2007. Molecular mechanisms of (−)-epicatechin and chlorogenic acid on the regulation of the apoptotic and survival/proliferation pathways in a human hepatoma cell line. J. Agric. Food Chem. 55, 2020–2027.

Gunzler, W., Kremers, H., Flohe, L., 1974. An improved coupled test procedure for glutathione peroxidase. Klin. Chem. Klin. Biochem. 12, 444–448.

Hecht, S., 1997. Approaches to cancer prevention based on an understanding *N*-nitrosamine carcinogenesis. Proc. Soc. Exp. Biol. Med. 216, 181–191.

Hissin, P., Hilf, R., 1976. A fluorometric method for determination of oxidised and reduced glutathione in tissues. Anal. Biochem. 74, 214–226.

Holley, S.L., Fryer, A.A., Haycock, J.W., Grubb, S.E.W., Strange, R.C., Hoban, P.R., 2007. Differential effects of glutathione *S*-transferase pi (GSTP1) haplotypes on cell proliferation and apoptosis. Carcinogenesis 28, 2268–2273.

Horder, M., Rej, R., 1984. Alanine aminotransferase. L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2. Routine UV method. In: Bergmeyer, H.V. (Ed.), Methods of Enzymatic Analysis. Verlag-Chemie, Weinheim, Germany, pp. 444–450.

Huynh, H., Nguyen, T.T.T., Chow, K.H.P., Tan, P.H., Soo, K.C., Tran, E., 2003. Overexpression of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular carcinoma: its role in tumor progression and apoptosis. BMC Gastroenterol. 3, 19.

Ito, Y., Sasaki, Y., Horimoto, M., Wada, S., Tanaka, Y., Kasahara, A., Ueki, T., Hirano, T., Yamamoto, H., Fujimoto, J., Okamoto, E., Hayashi, N., Hori, M., 1998. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. Hepatology 27, 951–958.

Jaeschke, H., Gores, G., Cederbaum, A., Hinson, J., Pessayre, D., Lemasters, J., 2002. Forum mechanisms of hepatotoxicity. Toxicol. Sci. 65, 166–176.

Kamaraj, S., Vinodhkumar, R., Anandakumar, P., Jagan, S., Ramakrishnan, G., Devaki, T., 2007. The effects of quercetin on antioxidant status and tumor markers in the lung and serum of mice treated with benzo(a)pyrene. Biol. Pharm. Bull. 30, 2268–2273.

Kang, J., Wanibuchi, H., Morimura, K., Gonzalez, F., Fukushima, S., 2007. Role of CYP2E1 in diethylnitrosamine-induced hepatocarcinogenesis *in vivo*. Cancer Res. 67, 11141–11146.

Köhle, C., Schwarz, M., Bock, K., 2008. Promotion of hepatocarcinogenesis in humans and animal model. Arch. Toxicol. 82, 623–631.

Lamuela-Raventós, R., Romero-Pérez, A., Andrés-Lacueva, C., Tornero, A., 2005. Review: health effects of cocoa flavonoids. Food Sci. Technol. Int. 11, 159–176.

Lecumberri, E., Goya, L., Mateos, R., Alía, M., Ramos, S., Izquierdo-Pulido, M., Bravo, L., 2007. A diet rich in dietary fiber from cocoa improves lipid profile and reduces malondialdehyde in hypercholesterolemic rats. Nutrition 23, 332–341.

Manso, M., Ramudo, L., De Dios, I., 2007. Extrapancreatic organ impairment during acute pancreatitis induced by bile-pancreatic duct obstruction. Effect of *N*-acetylcysteine. Int. J. Exp. Path. 88, 343–349.

Martin, M., Granado-Serrano, A., Ramos, S., Izquierdo-Pulido, M., Bravo, L., Goya, L., 2009. Cocoa flavonoids up-regulate antioxidant enzyme activity via the ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. J. Nutr. Biochem. doi: 10.1016/j.jnutbio.2008.10.009.

Martin, M., Ramos, S., Mateos, R., Granado-Serrano, A., Izquierdo-Pulido, M., Bravo, L., Goya, L., 2008. Protection of human HepG2 cells against oxidative stress by cocoa phenolic extract. J. Agric. Food Chem. 56, 7765–7772.

Masella, R., Di Benedetto, R., Vari, R., Filesi, C., Giovannini, C., 2005. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione-related enzymes. J. Nutr. Biochem. 16, 577–586.

Mates, J.M., Segura, J.A., Alfonso, J.F., Marquez, J., 2008. Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis and carcinogenesis. Arch. Toxicol. 82, 273–299.

Na, H., Surh, Y., 2008. Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. Food Chem. Toxicol. 46, 1271–1278.

Parekh, P., Rao, K.V.K., 2007. Overexpression of cyclin D1 is associated with elevated levels of MAP kinases, Akt and Pak1 during diethylnitrosamine-induced progressive liver carcinogenesis. Cell Biol. Int. 31, 35–43.

Principato, G.B., Aisa, M.C., Talesa, V., Rosi, G., Giovanini, E., 1985. Characterization of the soluble alkaline phosphatase from hepatopancreas of *Squilla mantis* L.. Comp. Biochem. Physiol. 80B, 801–804.

Ramakrishnan, G., Rao, H., Raghavendran, B., Vinodhkumar, R., Devaki, T., 2006. Suppression of *N*-nitrosodiethylamine induced hepatocarcinogenesis by silymarin in rats. Chem. Biol. Interact. 161, 104–114.

Ramiro-Puig, E., Casadesus, G., Lee, H., Zhu, X., McShea, A., Perry, G., Perez-Cano, F., Smith, M., Castell, M., 2009. Neuroprotective effect of cocoa flavonoids on in vitro oxidative stress. Eur. J. Nutr. 48, 54–61.

Ramiro-Puig, E., Urpí-Sardá, M., Pérez-Cano, F., Franch, A., Castellote, C., Andrés-Lacueva, C., Izquierdo-Pulido, M., Castell, M., 2007. Cocoa-enriched diet enhances antioxidant enzyme activity and modulates lymphocyte composition in thymus from young rats. J. Agric. Food Chem. 55, 6431–6438.

Ramos, S., 2007. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. J. Nutr. Biochem. 18, 427–442.

Ramos, S., 2008. Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. Mol. Nutr. Food Res. 52, 507–526.

Rej, R., Horder, M., 1984. Aspartate aminotransferase. L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.2.1. Routine UV method. In: Bergmeyer, H.V. (Ed.), Methods of Enzymatic Analysis. Verlag-Chemie, Weinheim, Germany, pp. 416–424.

Richert, S., Wehr, N., Stadtman, E., Levine, R., 2002. Assessment of skin carbonyl content as a noninvasive measure of biological age. Arch. Biochem. Biophys. 397, 430–432.

Schimtz, K.J., Wohlschlaeger, J., Lang, H., Sotiropoulos, G.C., Malago, M., Steveling, K., Reis, H., Cicinnati, V.R., Schmid, K.W., Baba, H.A., 2007. Activation of the ERK and AKT signaling pathway predicts poor prognosis in heptocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis virus infection. J. Hepatol. 48, 83–90.

Sen, N., Seaman, S., McPherson, M., 1980. Further studies on the occurrence of volatile and non-volatile nitrosamines in food. IARC Sci. Publ. 45, 7–460.

Sivaramakrishnan, V., Shilpa, P., Kumar, V., Devaraj, S., 2008. Attenuation of *N*-nitrosodiethylamine-induced hepatocellular carcinogenesis by a novel flavonol-Morin. Chem. Biol. Interact. 171, 79–88.

Sreepriya, M., Bali, G., 2005. Chemopreventive effects of embelin and curcumin against *N*-nitrosodiethylamine/phenobarbital-induced hepatocarcinogenesis in Wistar rats. Fitoterapia 76, 549–555.

Sreepriya, M., Bali, G., 2006. Effects of administration of embelin and curcumin on lipid peroxidation, hepatic glutathione antioxidant defense and hematopoietic system during N-nitrosodiethylamine/phenobarbital-induced hepatocarcinogenesis in Wistar rats. Mol. Cell. Biochem. 284, 49–55.

Sundaresan, S., Subramanian, P., 2008. Prevention of *N*-nitrosodiethylamine-induced hepatocarcinogenesis by S-allylcysteine. Mol. Cell. Biochem. 310, 209–214.

Tessitore, L., Bollito, E., 2006. Early induction of TGF-β1 through a fasting–re-feeding regimen promotes liver carcinogenesis by a sub-initiating dose of diethylnitrosamine. Cell Prolif. 39, 105–116.

Thorgeirsson, S., Grisham, J., 2002. Molecular pathogenesis of human hepatocellular carcinoma. Nat. Genet. 31, 339–346.

Vinson, J., Proch, J., Bose, P., Muchler, S., Taffera, P., Shutta, D., Samman, N., Agbor, G., 2006. Chocolate is a powerful ex vivo and in vitro antioxidant, antiatherosclerotic agent in an animal model, and a significant contributor to antioxidants in the European and American diets. J. Agric. Food Chem. 54, 8071–8076.

Vitaglione, P., Morisco, F., Caporaso, N., Fogliano, V., 2004. Dietary antioxidant compounds and liver health. Crit. Rev. Food Sci. Nutr. 44, 575–586.

Yadav, A., Bhatnagar, D., 2007. Chemo-preventive effect of Star anise in N-nitrosodiethylamine initiated and phenobarbital promoted hepato-carcinogenesis. Chem. Biol. Interact. 169, 207–214.

Yamagishi, M., Natsume, M., Nagaki, A., 2000. Antimutagenic activity of cacao: inhibitory effect of cacao liquor polyphenols on the mutagenic action of heterocyclic amines. J. Agric. Food Chem. 48, 5074–5078.

Yamagishi, M., Natsume, M., Osakabe, N., Nakamura, H., Furukawa, F., Imazawa, T., Nishikawa, A., Hirose, M., 2002. Effects of cacao liquor proanthocyanidins on PhIP-induced mutagenesis in vitro, and in vivo mammary and pancreatic tumorigenesis in female Sprague-Dawley rats. Cancer Lett. 185, 123–130.

Yamagishi, M., Natsume, M., Osakabe, N., Okazaki, K., Furukawa, F., Imazawa, T., Nishikawa, A., Hirose, M., 2003. Chemoprevention of lung carcinogenesis by cacao liquor proanthocyanidins in a male rat multi-organ carcinogenesis model. Cancer Lett. 191, 49–57.

Yoshida, H., Yoshida, H., Shiina, S., Omata, M., 2005. Early liver cancer: concepts, diagnosis, and management. Int. J. Clin. Oncol. 10, 384–390.