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1 # A diet rich in cocoa attenuates *N*-nitrosodiethylamine-
2 # induced liver injury in rats.

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16 **Running title:** Protective effects of cocoa in liver damage.
17
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21

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

28

29 **Abstract**

30

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

44

45 **Introduction**

46

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

54 Cocoa and its derived products are widely consumed in Europe and the United States
55 (Vinson, J. et al., 2006) and they have increasingly attracted researchers, food manufacturers,
56 as well as consumers due to their biological properties, which have been mostly related to its
57 phenolic compounds (Lamuela-Raventós, R. et al., 2005). Cocoa flavonoids can influence
58 several important biological functions by their free-radical scavenging ability or through the
59 regulation of signal transduction pathways to stimulate apoptosis and/or to inhibit
60 inflammation and proliferation in different human cancer cell lines (Lamuela-Raventós, R. et
61 al., 2005, Martin, M. et al., 2009, Martin, M. et al., 2008, Ramos, S., 2007, Ramos, S., 2008).
62 Therefore, cocoa phenolic compounds have been suggested not only as potential protective
63 and even therapeutic agents to reduce liver damage (Ramos, S., 2008, Vitaglione, P. et al.,
64 2004).

65 The modulation of phase I [glutathione reductase (GR), glutathione peroxidase (GPx) and
66 catalase (CAT)] and phase II [glutathione-S-transferase (GST)] enzymes, and gluthatione
67 (GSH) levels play a primary role in the balance of the redox status through the reduction of
68 reactive oxygen spices (ROS) and peroxides produced in the organism, as well as in the
69 detoxification of xenobiotics (Ramiro Puig, E. et al., 2007, Ramos, S., 2008). Cocoa has been

70 shown to enhance the activity of phase I enzymes in hepatic and thymocyte cells, respectively
71 (Martin, M. et al., 2009, Ramiro Puig, E. et al., 2007), and it may also induce cell survival and
72 proliferation, as well as protective effects, through the modulation of different transduction
73 pathways, such as AKT and MAPKs (Martin, M. et al., 2009, Ramiro-Puig, E. et al., 2009).
74 Additionally, upregulation of phase I and II enzymes mediated by MAPKs and/or AKT has
75 been reported for different polyphenols (Masella, R. et al., 2005, Na, H. and Surh, Y., 2008).
76 In this regard, induction of GPx and GR by cocoa has been shown to occur via ERK in
77 hepatic cells (Martin, M. et al., 2009). Moreover, GST expression has been implicated in the
78 regulation of cell proliferation and protection from apoptosis through JNK-mediated
79 mechanisms (Holley, S.L. et al., 2007, Mates, J.M. et al., 2008).
80 *N*-nitrosodiethylamine (DEN) is a potent hepatotoxic, carcinogen and mutagen (Chuang, S. et
81 al., 2000, Köhle, C. et al., 2008, Sreepriya, M. and Bali, G., 2006, Tessitore, L. and Bollito,
82 E., 2006). Human exposure could occur through the diet (meat, whiskey, etc.) (Hecht, S.,
83 1997, Sen, N. et al., 1980), in certain occupational settings, smoking or through the use of
84 cosmetics, pharmaceutical products and agricultural chemicals (Hecht, S., 1997). DEN has
85 been extensively used as an initiating carcinogen in experimental animal models (Chuang, S.
86 et al., 2000, Köhle, C. et al., 2008, Ramakrishnan, G. et al., 2006, Sivaramakrishnan, V. et al.,
87 2008, Sreepriya, M. and Bali, G., 2005, Sreepriya, M. and Bali, G., 2006, Sundaresan, S. and
88 Subramanian, P., 2008, Tessitore, L. and Bollito, E., 2006) and induces hepatic necrosis
89 through metabolic activation by CYP2E1 in experimental animals (Kang, J. et al., 2007).
90 Activation of DEN, which takes place mainly in liver microsomes (Kang, J. et al., 2007), has
91 been shown to stimulate Kupfer cells leading to generate high levels of ROS, capable of
92 damaging liver cells and participating in the induction of hepatocarcinogenesis (Kang, J. et
93 al., 2007). It has been shown that a cocoa bean preparation protects against different types of
94 cancers (Yamagishi, M. et al., 2002, Yamagishi, M. et al., 2003), as well as against a number

95 of hepatotoxic agents including nitrosamines (Amin, I. et al., 2004, Yamagishi, M. et al.,
96 2000). However, the mechanisms by which cocoa elicits such hepatoprotective and
97 chemopreventive effects related to antioxidant defences, as well as survival/proliferation
98 pathways, remain poorly understood.
99 Thus, the aim of this study was to evaluate the protective effect of a cocoa-rich diet against
100 DEN-induced liver toxicity. Here, we report that cocoa partly attenuates DEN-induced liver
101 injury in rats via induction of antioxidant defence (GSH, CAT, GPx and GST) and
102 modulation of signals related to cell death (caspase-3 and JNK) and survival/proliferation
103 (AKT).
104

105 **Materials and Methods**

106

107 *Materials and chemical*

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

116

117 *Cocoa*

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

122

123 *Animal treatments*

124 Forty five male Sprague-Dawley rats (6 weeks old) were obtained from the School of
125 Medicine, Universidad Autónoma (Madrid, Spain). Animals were placed individually in
126 stainless steel wire-bottomed metabolic cages housed in a room under controlled conditions
127 (19-23 ºC, 50-60 % humidity and 12 h light/darkness cycles).

128 Diets were prepared from a Fibre Free AIN-93M Purified Rodent Diet (Panlab S.L.,
129 Barcelona, Spain) providing all nutrients required by adult rats. Rats were randomly assorted
130 into five different experimental groups (eight to ten animals per group) (Figure 1) and were

131 provided with food and water *ad libitum*. Initially, three groups received a standard diet (C,
132 groups 1, 3 and 5) and two groups were fed with cocoa-rich diet (Ca, groups 2 and 4),
133 containing the basal diet supplemented with 16 % powdered cocoa (providing 54.4 g of
134 dietary fibre per Kg of diet), added as a source of polyphenols. In line with our previous
135 research, this powdered supplement was formulated to provide 7.5 g of polyphenols per Kg of
136 diet, since this percentage of cocoa supplementation improve the antioxidant defence system,
137 the lipid profile and reduced the lipid peroxidation. (Lecumberri, E. et al., 2007). The
138 composition of the diets is given in Table 1 and cocoa powder contains 26% proteins and 12%
139 lipids.
140 To induce hepatic damage, three groups of rats (groups 3-5) were injected intraperitoneally
141 with 200 mg/Kg DEN diluted in saline on days 14 and 28. Rats in the non-DEN treated
142 groups were injected with saline the same day. In addition, on day 28, group 5 was changed
143 from control diet to cocoa diet after the second DEN injection (Figure 1) to test the potential
144 therapeutic activity of the cocoa-rich diet.
145 Animal weight and food intake were monitored daily for 6 weeks. Blood was harvested from
146 the trunk after decapitation, and serum was separated by centrifugation at 1000 xg, 10 min,
147 4ºC for further biochemical analysis. Livers were collected, weighted and frozen in liquid N_2
148 and stored at –80ºC.
149 Animals were treated according to the Institutional Care Instructions (Bioethical Commission
150 from Consejo Superior de Investigaciones Cientificas).
151
152 *Analysis of hepatic enzymes (AST, ALT, ALP)*
153 As markers for liver function in serum, the activity of aspartate transaminase (AST), alanine
154 transaminase (ALT) and alkaline phosphatase (ALP) [expressed as Units (U)/L serum] were
155 analysed. Serum AST was measured spectrophotometrically at 340 nm in the presence of α-

156 ketoglutarate, aspartate, NADH and malate dehydrogenase, following the method of Rej and
157 Horder (Rej, R. and Horder, M., 1984). Serum ALT was assayed at 340 nm in the presence of
158 α-ketoglutarate, pyruvate, NADH and lactate dehydrogenase as described by Horder and Rej
159 (Horder, M. and Rej, R., 1984). Serum ALP was analysed spectrophotometrically at 405 nm
160 using the formation of *p*-nitrophenol from *p*-nitrophenylphosphate as a substrate (Principato,
161 G.B. et al., 1985). Serum protein concentration was measured by the Bradford reagent.
162
163 *Determination of GSH*
164 The content of GSH was quantitated by the fluorometric assay of Hissin and Hilf (Hissin, P.
165 and Hilf, R., 1976). The method takes advantage of the reaction of GSH with *o*-
166 phthalaldehyde (OPT) at pH 8.0, which generates fluorescence.
167 Livers were homogenized (1:20 w/v) in 50 mM phosphate buffer pH 7.0, proteins precipitated
168 with 5% trichloroacetic acid and then centrifuged for 30 min at 10.000 xg. Following, 50 µL
169 of the clear supernatant were transferred to a 96 multiwell plate for the assay. Fluorescence
170 was measured at an excitation wavelength of 340 nm and emission wavelength of 460 nm.
171 The results were interpolated in a glutathione standard curve (5 ng-1 µg) and expressed as
172 nmol GSH per milligram of protein, which was determined by the Bradford reagent. The
173 precise protocol has been described elsewhere (Alia, M. et al., 2003, Alía, M. et al., 2006).
174
175 *Determination of GPx, GR, CAT and GST activities*
176 The activity of antioxidant enzymes (GPx, GR and catalase) was determined in liver
177 homogenates. Livers (0.3 g) were homogenized (1:5 w/v) in 0.25 M Tris, 0.2 M sucrose and
178 5 mM 1,4-dithiothreitol (DTT) buffer pH 7.4 and centrifuged at 3.000 xg for 15 min.
179 Determination of GPx activity is based on the oxidation of GSH by GPx, using t-BOOH as a
180 substrate, coupled to the disappearance of NADPH by GR (Gunzler, W. et al., 1974),

181 expressing the results as mU/mg protein. GR activity was determined by following the
182 decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized
183 glutathione (Goldberg, D. and Spooner, R.). The GR activity was expressed as μU/mg
184 protein. Catalase (CAT) activity was determined by following the decomposition of H_2O_2
185 measured as a decrease in absorbance at 240 nm (Aebi, H., 1987) and expressed as UI/mg
186 protein. The methods have been previously described (Alia, M. et al., 2003, Alía, M. et al.,
187 2006).
188 Analysis of GST was carried out in liver and serum by the Biovision Commercial kit GST
189 fluorometric activity assay (Biovision, Mountain View, CA) and the activities were expressed
190 as U/mg protein and mU/mg protein, respectively. Protein was measured by the Bradford
191 reagent.
192
193 *Protein carbonyl determination in liver*
194 Protein oxidation of liver homogenates was measured as carbonyl groups content according to
195 the method of Richert *et al*. (Richert, S. et al., 2002). Absorbance was measured at 360 nm
196 and carbonyl content was expressed as nmol/mg protein using an extinction coefficient of
197 22000 nmol L^{-1} cm^{-1}. Protein in liver homogenates was determined by the Bradford reagent.
198
199 *Lactate Dehydrogenase (LDH) Leakage Assay*
200 Livers were homogenized (1:5 w/v) in 50 mM phosphate buffer (PBS) pH 7.5, centrifuged for
201 30 min at 1000 xg and the supernatants collected. LDH was spectrophotometrically assayed
202 in the samples by measuring the disappearance of NADH at 340 nm, as previously described
203 (Alía, M. et al., 2006, Bergmeyer, H. and Bernt, E., 1974). The reaction mixture contained 5
204 mM piruvate, 0.35 mM NADH, 84 mM Tris and 50 μg of protein per condition. Enzyme
205 activity was calculated by using an extinction coefficient of 6.22 mM^{-1} cm^{-1} and expressed in

206 units using mg^{-1} protein. One unit of the enzyme activity is defined as the amount of enzyme
207 required to oxidize 1 µmol NADH min^{-1}.
208
209 *Liver caspase-3 activity*
210 Activation of caspase-3 was determined as previously described (Granado-Serrano, A. et al.,
211 2007). Briefly, livers were lysed (1:5 w/v) in a buffer containing 5 mM Tris (pH 8), 20 mM
212 EDTA, and 0.5 % Triton X-100. The reaction mixture contained 20 mM HEPES (pH 7), 10 %
213 glycerol, 2 mM DTT, 50 µg of protein per condition and 20 µM Ac-DEVDAMC (*N*-acetyl-
214 Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) as substrate. Enzymatic activity was
215 determined by measuring fluorescence at an excitation wavelength of 380 nm and an emission
216 wavelength of 440 nm (Bio-Tek, Winooski, VT, USA).
217
218 *Preparation of cell lysates for Western blotting*
219 Samples of frozen liver were homogenized 1:10 (w:v) in extraction buffer [50 mM HEPES
220 (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 % glycerol, 10 mM $Na_4P_2O_7$, 10 mM
221 NaF, 2 mM EDTA, 1 % Nonidet P-40, 2 mM Na_3VO_4, 5 µg/mL leupeptin, 20 µg/mL
222 aprotinin, 2 mM benzamidin and 2 mM phenylmethylsulphonyl fluoride (PMSF)] to detect
223 AKT, p-AKT, ERK1/2, p-ERKs, JNK1/2 and p-JNKs (Gavete, L. et al., 2005). Homogenates
224 were centrifuged at 14000 xg for 60min and the supernatants were collected, assayed for
225 protein concentration by using the Bradford reagent, aliquoted and stored at –80 ºC until use
226 for Western blot analyses.
227
228 *Protein determination by Western Blotting*
229 Equal amounts of protein (100 µg) were separated by SDS-PAGE and transferred to
230 polyvinylidene difluoride (PVDF) filters (Protein Sequencing Membrane, BioRad).
231 Membranes were probed with the corresponding primary antibody followed by incubation

232 with peroxide-conjugated antirabbit Ig (GE Healthcare, Madrid, Spain). Blots were developed
233 with the ECL system (GE Healthcare, Madrid, Spain). Normalization of Western blot was
234 ensured by ß-actin and band quantification was carried out with a scanner and the Scion
235 Image software.

236

237 *Statistics*

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

242

243 **Results**

244

245 *Body and liver weights and food consumption*

246 Final body weights and food consumption decreased after DEN administration in all groups
247 of animals (groups 3-5) (Table 2). Consistent with these results, body weight gains of groups
248 1 and 2 were higher than those in DEN-injected groups. The lowest values were observed in
249 group 5 corresponding to DEN-treated animals that received the cocoa-rich diet at day 28,
250 after being fed with standard diet (Table 2). Therefore, the food efficiency diminished in
251 groups 3-5 when compared to DEN-untreated animals (groups 1 and 2), and the smallest
252 alimentary efficiencies were observed in DEN-rats receiving the cocoa-rich diet (groups 4 and
253 5).
254 Liver weights of group 5 were diminished when compared to all other groups of animals.
255 However, no significant differences in liver-to-body weight ratio were observed among the
256 experimental groups (Table 2).

257

258 *Levels of markers of liver function in serum*

259 As shown in Table 3, the serum total protein levels were reduced in DEN-treated rats, and
260 were not normalized by the cocoa supplementation; on the contrary, group 5, in which diet
261 was changed from standard to cocoa-rich one, showed the lowest values (Table 3).
262 The serum activity of ALT exhibited an elevation in DEN-treated groups and remained
263 increased in animals receiving cocoa-diet in comparison with untreated rats (Table 3).
264 Addition of cocoa to DEN-injected animals (groups 4 and 5) showed an enhanced activity of
265 AST when compared to untreated (groups 1 and 2) and DEN-treated (group 3) rats. Similarly,
266 ALP activity was increased because of the administration of DEN in all experimental groups

267 (groups 3-5). However, only in cocoa-fed rats treated with DEN (group 4), an increase in
268 GST activity was observed (Table 3).
269
270 *Levels of liver GSH and phase I and II enzymes*
271 Liver GSH concentration decreased by DEN administration in group 3, which showed the
272 lowest levels (Figure 2). However, liver GSH content was elevated by cocoa in control and
273 DEN-treated rats (groups 2 and 4, Figure 2), whereas animals fed with cocoa-diet for 2 weeks
274 (group 5) displayed similar values to control DEN-untreated rats (group 1).
275 Similar results were obtained for GPx activity, with increased values in the animals
276 consuming cocoa for 6 weeks (groups 2 and 4), decreased GPx activity after DEN treatment
277 in rats fed with standard diet (group 3), and recovering of control values in group 5. GR
278 showed an increased activity in untreated animals fed cocoa (group 2), while all the DEN-
279 treated groups showed similar values to controls (group 1) (Figure 2). Similarly to GPx,
280 catalase activity decreased in animals fed with standard diet and treated with DEN (group 3),
281 whereas the other groups of rats displayed similar levels to controls (Figure 2). GST activity
282 in liver remained unaltered in all animals except in group 4, where an increase in its activity
283 was detected (Figure 2). Therefore, cocoa induces the antioxidant defence in the liver of the
284 control and DEN-injected rats.
285
286 *Protein carbonyl content and LDH levels and caspase-3 activity in liver*
287 Levels of protein carbonyl content were increased in group 3 due to DEN administration
288 when compared to no-injected animals (Figure 3). However, cocoa-fed rats treated with DEN
289 (groups 4 and 5) showed a decreased protein carbonyl levels when compared to group 3,
290 values were reverted to control levels in group 4 (cocoa-fed during 4 weeks) or partly
291 diminished in group 5 (cocoa-fed for 2 weeks).

292 Lactate dehydrogenase levels were increased by DEN administration in all groups (groups 3-
293 5), pointing at a DEN-induced liver injury that was not prevented by the cocoa-rich diet
294 (Figure 3). DEN also induced caspase-3 activity (group 3), but cocoa administration was
295 capable of returning caspase-3 activity to control levels in animals consuming the cocoa-rich
296 diet throughout the experiment (group 4), or partly decreased the DEN-induced activation of
297 caspase-3 in animals receiving the cocoa-rich diet only for two weeks (group 5). Animals fed
298 with the cocoa-rich diet (group 2) showed LDH levels similar to control rats and decreased
299 values of caspase-3 activity (Figure 3). These results indicate that cocoa diminished the
300 oxidative (carbonyl protein content) and apoptotic damages (caspase-3 activation), but not the
301 necrotic DEN-induced liver injury (LDH activity).
302
303 *Liver AKT, ERK and JNK levels*
304 Figures 4 and 5 illustrate that DEN-treated animals fed with the standard diet (group 3)
305 showed a significant increase in the levels of phosphorylated AKT and JNK proteins, whereas
306 no changes in these two proteins were found in the other groups of animals. Phosphorylated
307 ERK levels were increased by cocoa administration in control animals (group 2), but they
308 were dramatically decreased by DEN treatment in all injected rats (groups 3-5) (Figure 5).
309 There was no difference in the total levels of AKT, ERKs and JNKs among the groups. Thus,
310 cocoa prevented the activation of AKT and JNK, although it did not modify the decreased
311 values of active ERK induced by DEN.
312

313 **Discussion**

314

315 DEN toxicity is primarily associated to an excessive production of free radicals in the liver.
316 As a consequence, reactive electrophilic intermediates are formed, which overwhelms the
317 antioxidant defences and ultimately proceeds to oxidative stress paving way to liver damage
318 (Kang, J. et al., 2007). DEN induces a postnecrotic hepatocellular proliferation that
319 contributes to enhance the number of initiated cells (Cascales, M., 2001), and it is accepted as
320 a model to study the relations among liver necrosis, cancer initiation and replication
321 (Cascales, M., 2001, Chuang, S. et al., 2000, Köhle, C. et al., 2008, Ramakrishnan, G. et al.,
322 2006, Sivaramakrishnan, V. et al., 2008, Sreepriya, M. and Bali, G., 2005, Sreepriya, M. and
323 Bali, G., 2006, Sundaresan, S. and Subramanian, P., 2008, Tessitore, L. and Bollito, E.,
324 2006).
325 Cocoa and its derived products have been demonstrated to contain important antioxidant
326 polyphenols that inhibit different tumoral processes and exhibit antioxidant and anti-
327 inflammatory properties (Amin, I. et al., 2004, Lamuela-Raventós, R. et al., 2005, Martin, M.
328 et al., 2009, Martin, M. et al., 2008, Yamagishi, M. et al., 2000, Yamagishi, M. et al., 2002,
329 Yamagishi, M. et al., 2003). In this study, we show that a cocoa-rich diet partly protect the
330 liver against an oxidative-induced damage by decreasing the protein carbonyl content and
331 modulating the activities of antioxidant enzymes and by regulating key proteins of cell
332 signalling cascades, although cocoa-rich diet was unable to abrogate the DEN-induced
333 enhanced values of hepatospecific enzymes and LDH. The cocoa dose was finally selected
334 based on our own previous in vivo studies, where it is showed that it contributed to reduce the
335 cardiovascular risk acting as an effective antioxidant (Lecumberri, E. et al., 2007).
336 Reduction of food intake and consequently, the reduction of body weight gain observed in
337 DEN-treated animals, could be largely due to losses from skeletal muscle and adipose tissue

338 as previously shown (Sreepriya, M. and Bali, G., 2005), and it could be considered as an
339 indirect indication of the declining hepatic function following exposure to DEN (Amin, I. et
340 al., 2004, Ramakrishnan, G. et al., 2006, Sivaramakrishnan, V. et al., 2008, Sreepriya, M. and
341 Bali, G., 2005). In addition, assessment of the liver/body weights ratio was used to investigate
342 potential changes in the liver size, but no differences were found (Ramakrishnan, G. et al.,
343 2006, Sreepriya, M. and Bali, G., 2005, Yamagishi, M. et al., 2003).
344 Low levels of albumin have been reported in the serum of patients and animals with early
345 hepatocellular cancer (Sreepriya, M. and Bali, G., 2005, Yoshida, H. et al., 2005). Contrary to
346 other antioxidants such as curcumin and embelin that reverted the diminished serum proteins
347 values induced by DEN (Sreepriya, M. and Bali, G., 2005), our results indicate that cocoa did
348 not exhibit its protective effects during DEN-induced liver damage by enhancing the total
349 protein levels.
350 GSH is an important non-enzymatic antioxidant defence required to maintain the normal
351 redox state of cells and to counteract deleterious effects of oxidative stress. GSH depletion
352 ultimately promotes oxidative stress, with a cascade of effects thereby affecting functional
353 and structural integrity of cell and organelle membrane (Masella, R. et al., 2005). Our results
354 showed that the significant decrease in GSH levels in animals exposed to DEN was restored
355 or enhanced by cocoa feeding, which indicated the potentiality of cocoa to counteract the
356 oxidative damage induced by DEN and to reinforce the antioxidant defence in normal
357 conditions, as previously reported for other natural antioxidants (Ramakrishnan, G. et al.,
358 2006, Sivaramakrishnan, V. et al., 2008, Sreepriya, M. and Bali, G., 2006).
359 Chemical induction of liver damage by DEN administration is associated with changes in the
360 oxygen radical metabolism in this organ, which were demonstrated by measuring protein
361 carbonyl content and the activity of the antioxidant enzymes. Generation of protein, DNA and
362 lipid oxidation products by DEN administration at the initiation stage can be prevented by

363 natural antioxidants (Ramakrishnan, G. et al., 2006, Sivaramakrishnan, V. et al., 2008,
364 Sreepriya, M. and Bali, G., 2006). Thus, DEN-treated rats fed with cocoa (groups 4 and 5)
365 displayed lower levels of protein carbonyl content when compared with rats injected with
366 DEN receiving regular diet (group 3). This reveals the protective role of cocoa against the
367 induced-oxidative stress (Lecumberri, E. et al., 2007, Martin, M. et al., 2008, Ramiro-Puig, E.
368 et al., 2009).

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

381 GST catalyses the reaction of endogenous GSH with numerous electrophiles to yield less
382 toxic conjugates that are easily eliminated (Masella, R. et al., 2005). Although increased and
383 decreased liver and serum GST activities have been reported after DEN-induced damage
384 (Amin, I. et al., 2004, Sivaramakrishnan, V. et al., 2008, Sreepriya, M. and Bali, G., 2006,
385 Sundaresan, S. and Subramanian, P., 2008, Yadav, A. and Bhatnagar, D., 2007), we found
386 that liver and serum GST activities increased in rats fed with cocoa and injected with DEN
387 (group 4), suggesting that cocoa administration contributes to the liver protection against the

388 oxidative induced-injury. In this regard, it has been demonstrated that different natural
389 compounds and polyphenols, as well a cocoa bean product, induce GST as one of the
390 principal anticarcinogenic mechanisms (Amin, I. et al., 2004, Masella, R. et al., 2005,
391 Sundaresan, S. and Subramanian, P., 2008). Moreover, some studies point to the induction of
392 GST as a mechanism to protect against chemically induced cancer and oxidative stress by
393 increasing the metabolism of electrophilic intermediates and ROS (Masella, R. et al., 2005).
394 Enhancement of liver LDH activity, a sensitive marker of hepatocyte injury, indicates a
395 nonspecific alteration in the plasma membrane integrity and permeability and/or may be due
396 to its overproduction by tumour cells (Kamaraj, S. et al., 2007, Manso, M. et al., 2007,
397 Sivaramakrishnan, V. et al., 2008). The significant DEN-induced increase in serum hepatic
398 marker enzymes and liver LDH activities that indicated the hepatocellular dysfunction and the
399 severity of the liver necrotic damage (Amin, I. et al., 2004, Kamaraj, S. et al., 2007,
400 Sivaramakrishnan, V. et al., 2008), were not avoided by the administration of a cocoa-rich
401 diet.
402 Activation of caspase-3, the most important enzyme responsible for apoptosis, has been
403 considered as a sensitive method of detecting liver damage and has been associated with
404 progressive liver fibrosis (Bantel, H. et al., 2004). Moreover, high rates of apoptosis have
405 been demonstrated in liver cancer (Kang, J. et al., 2007, Schimtz, K.J. et al., 2007). In
406 addition, it should be mentioned that administration of DEN (200 mg/Kg) causes fibrosis and
407 necrosis in the liver (Tessitore, L. and Bollito, E., 2006). It is noticeable that DEN-injected
408 rats fed with standard diet (group 3) showed levels of AST and ALT that were below those of
409 the other DEN-treated groups. This finding could be a consequence of a reduction in the
410 number of viable hepatocytes due to enhanced cell death in liver, as these animals also
411 showed the highest levels of LDH and caspase-3 (Manso, M. et al., 2007). Thus, the
412 decreased caspase-3 activity in animals not injected with DEN and fed with cocoa (group 2),

413 as well as in DEN-treated rats receiving cocoa (groups 4 and 5), suggested a potential
414 protective effect of cocoa against cell death in normal and under oxidative conditions.
415 Therefore, although additional studies are needed, it could be suggested that cocoa could
416 partly protected hepatocytes against the progression of the fibrotic damage induced by DEN.
417 The signalling mechanisms associated with liver damage induced by oxidative-stress are not
418 completely known. In our study, the increase in p-AKT levels in DEN-treated rats fed the
419 standard diet (group 3) agrees with that reported in animals receiving DEN in drinking water
420 (Parekh, P. and Rao, K.V.K., 2007). These data support the important role of AKT in
421 controlling the balance between survival and apoptosis (Ramos, S., 2008), which has been
422 considered a critical factor in the aggressiveness of hepatocellular cancer (Parekh, P. and Rao,
423 K.V.K., 2007, Schimtz, K.J. et al., 2007). Moreover, the value of p-AKT was restored in rats
424 injected with DEN but fed cocoa (groups 4 and 5) suggests that cocoa prevents the activation
425 of this main protein related to cell survival/proliferation; this could result in a potential
426 attenuation of the postnecrotic proliferation induced by DEN and in a reduction of the number
427 of initiated cells (Cascales, M., 2001).
428 ERK plays a critical role in controlling the balance between cell survival and proliferation,
429 and cell cycle progression (Schimtz, K.J. et al., 2007). Although data on p-ERK values
430 remain controversial (Ito, Y. et al., 1998, Parekh, P. and Rao, K.V.K., 2007), increased p-
431 ERK levels have recently been related to an advanced, but not early, tumour stage (Huynh, H.
432 et al., 2003). This finding supports our results, since decreased levels of p-ERK were found in
433 all DEN-injected animals, a model to study the relations among necrosis and cancer initiation.
434 Moreover, p-ERK was increased in rats fed a cocoa-rich diet indicating that cocoa
435 components can activate cellular kinases (Ramos, S., 2008) in concert with our previous
436 results (Granado-Serrano, A. et al., 2007, Martin, M. et al., 2009, Ramiro-Puig, E. et al.,
437 2009). Similarly, the enhanced levels of p-ERK in cocoa fed rats (group 2) could be related to

438 their increased GR and GPx activities, since MAPKs pathway has recently been implicated in
439 the up-regulation of several antioxidant enzymes activities in liver cancer cells (Martin, M. et
440 al., 2009).

441 The JNK family belongs to the MAPKs superfamily (same as ERKs) and its activation has
442 been associated to apoptosis in liver (Czaja, M., 2003, Parekh, P. and Rao, K.V.K., 2007).
443 DEN administration enhanced the p-JNK/JNK ratio in animals fed with the standard diet
444 (group 3) and returned to control values in DEN-groups receiving cocoa (groups 4 and 5). In
445 line with this, a protective effect of cocoa flavonoids has been described on a model of
446 oxidative stress in neurons, showing a down-regulation of p-JNK levels (Ramiro-Puig, E. et al.,
447 2009). Moreover, DEN-treated rats fed with regular diet showed the highest LDH and caspase-
448 3 activities and p-JNK/JNK values, pointing out to an enhanced cell death in the liver of these
449 animals, although this group also exhibited enhanced p-AKT values suggestive of a promotion
450 of cellular survival/proliferation. At the moment we cannot explain this finding and further
451 studies are needed to elucidate this result, but similar effects have been reported in patients
452 with liver cancer (Kang, J. et al., 2007, Schimtz, K.J. et al., 2007).

453 Finally, it is important to mention that in vitro studies have attributed the protective effect of
454 cocoa to the polyphenols, since the contribution of theobromine was considered negligible
455 (Martin, M. et al., 2008). Additionally, a similar protective effect has been recently described
456 after treating cells with a cocoa phenolic extract or (-)-epicatechin (Ramiro-Puig, E. et al.,
457 2009). Therefore, it could be suggested that the beneficial effects of cacao in the present study
458 might be ascribed to the polyphenolic compounds, which even may develop synergism among
459 them, but further studies are needed.

460 In summary, a cocoa-rich diet protects cells against DEN-induced oxidative stress by activating
461 the antioxidant defence system. In addition, cocoa appears to exert an antiapoptotic effect in
462 the liver of DEN-treated animals that could be associated with the prevention of JNK and

463 caspase-3 activation. Cocoa also prevented the DEN-induced increase of the
464 survival/proliferation signal AKT, associated with poor prognosis in liver cancer. All these
465 effects contribute to attenuate the liver DEN-induced oxidative damage. However, cocoa was
466 not completely capable of avoiding the liver necrotic damage induced by the hepatotoxic as
467 shown by the increased activities of the hepatospecific enzymes and LDH as well as decreased
468 p-ERK values. These results provide new insights into the antioxidative mechanisms of cocoa
469 flavonoids and point towards their antiapoptotic and modulatory effects on cell death and
470 survival/proliferation pathways as additional mechanisms of action of these compounds.
471 Therefore, it could be suggested that cocoa or cocoa products enriched in flavonoids, which
472 lack of toxicity in humans, may contribute to the protection against liver oxidative stress-
473 related diseases.
474

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476

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480 The authors declare that there are no conflicts of interest.

481

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639

640 **Figure Captions**

641

642 Figure 1. Schematic representation of the experimental design used for the study.

643

644 Figure 2. Effect of cocoa on (A) glutathione levels and activity of (B) GPx, (C) GR, (D) CAT
645 and (E) GST liver from control and DEN groups fed with standard (C) and cocoa-rich (Ca)
646 diets. Data represent the means ± SD (n=8-10). Means without a common letter differ, $P<$
647 0.05.

648

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

652

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

658

659 Figure 5. Effects of cocoa on the basal levels of phosphorylated ERK1/2 (Thr202/Tyr204),
660 total ERK1/2, phosphorylated JNK1/2 (Thr183/Tyr185) and total JNK in controls and DEN-
661 treated animals. (A) Representative blots of both MAPKs. Percentage data of (B) p-
662 ERK/ERK and (C) p-JNK/JNK ratios relative to controls (means ± SD, n=7). The same liver
663 homogenates were subjected to Western blot analysis using the corresponding non-phospho-

664 specific antibodies to detect total ERK or JNK. Equal loading of Western blots was ensured

665 by β-actin. Means without a common letter differ, $P< 0.05$.

666

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

	C	Ca
Casein	140	140
Dextrose	155	155
Sucrose	100	100
Fat	40	40
t-BHQ[1]	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

669

670 [1] *tert*-butylhydroquinone

671

672 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*.

	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 42d)	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}

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

686 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*.

687

	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]

688 689 690 691 692 693 694 695

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

697 **Figure 1.**
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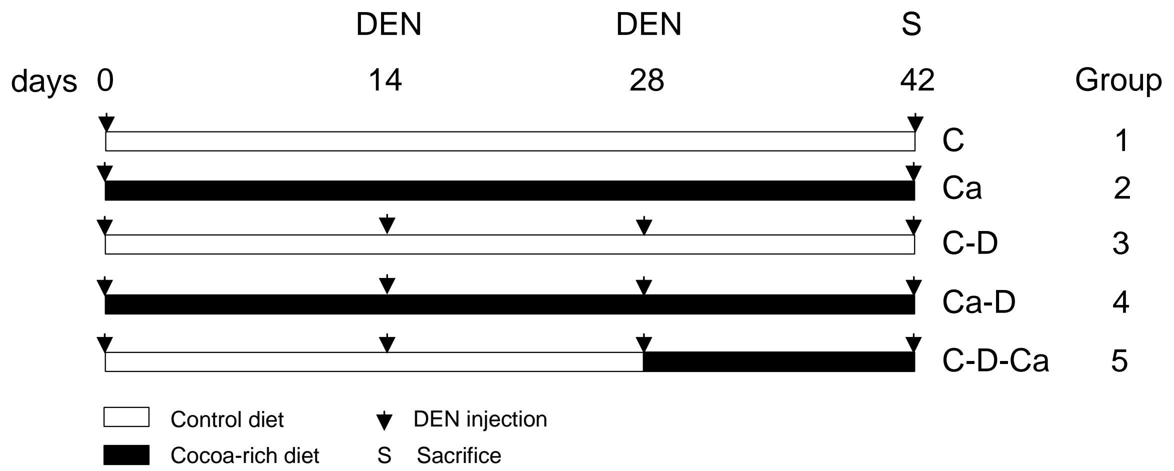
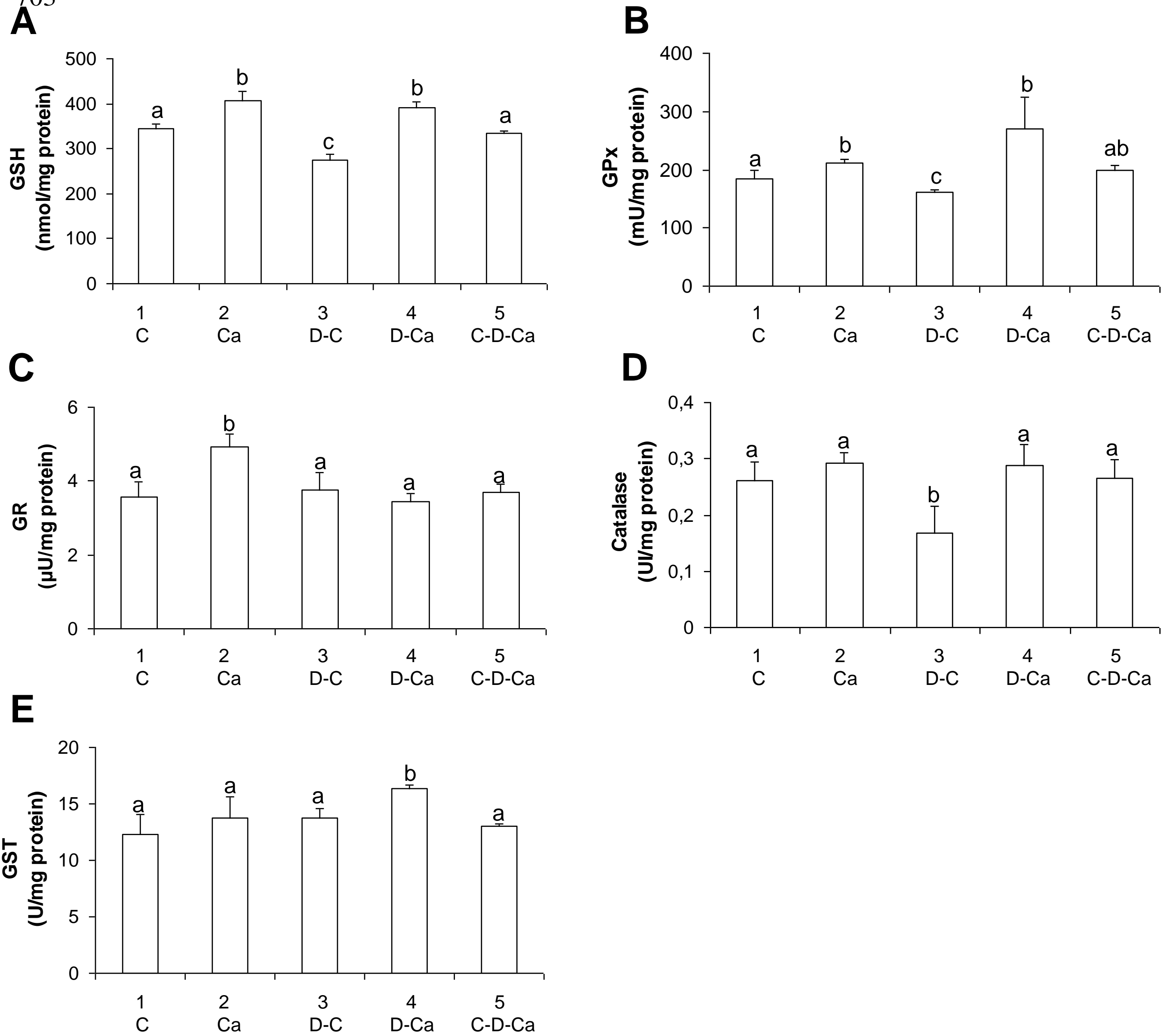


702 **Figure 2.**
703



704 **Figure 3.**
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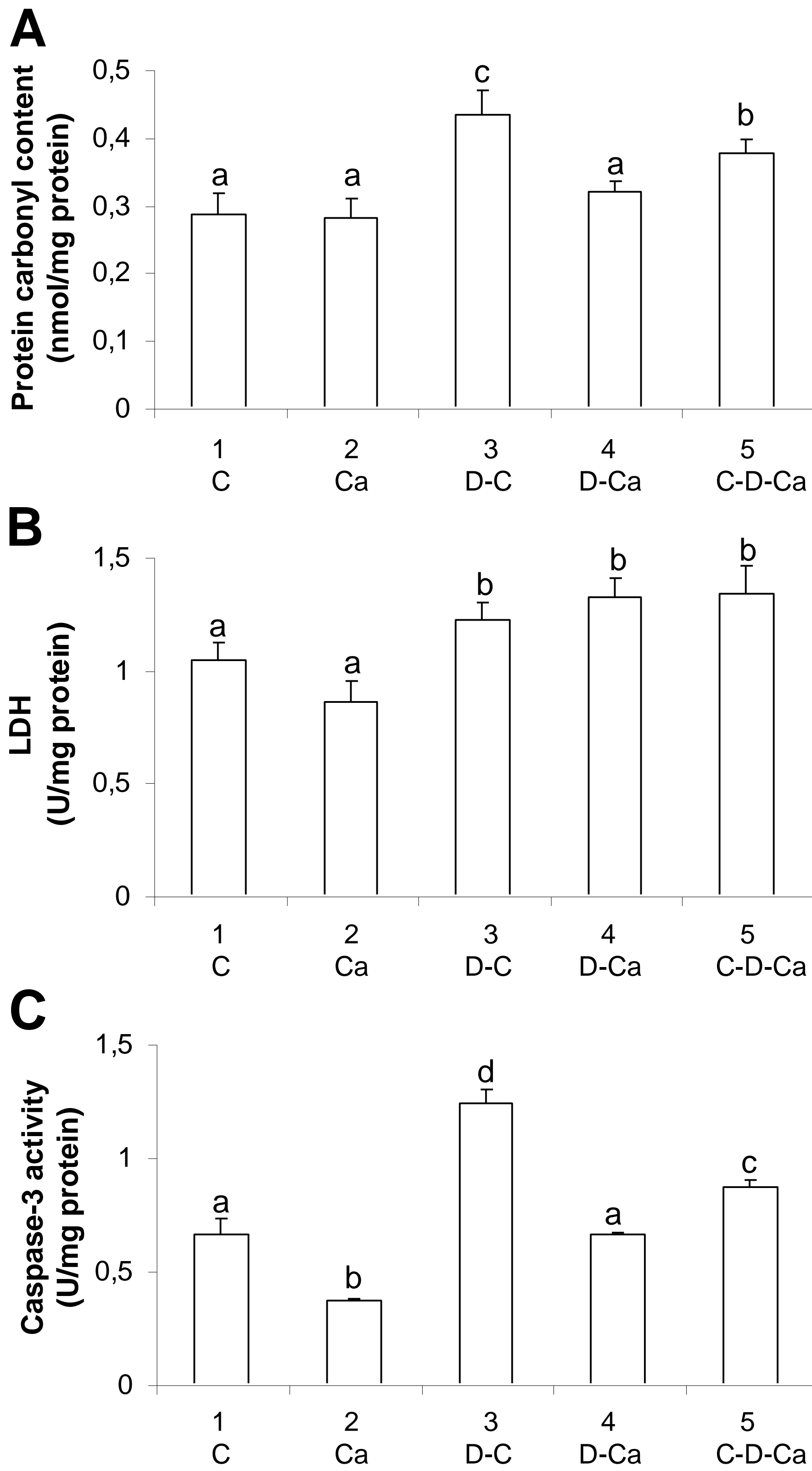
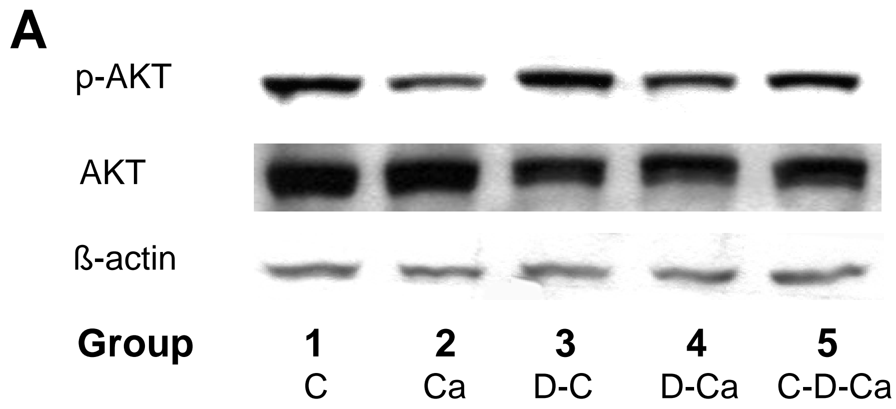
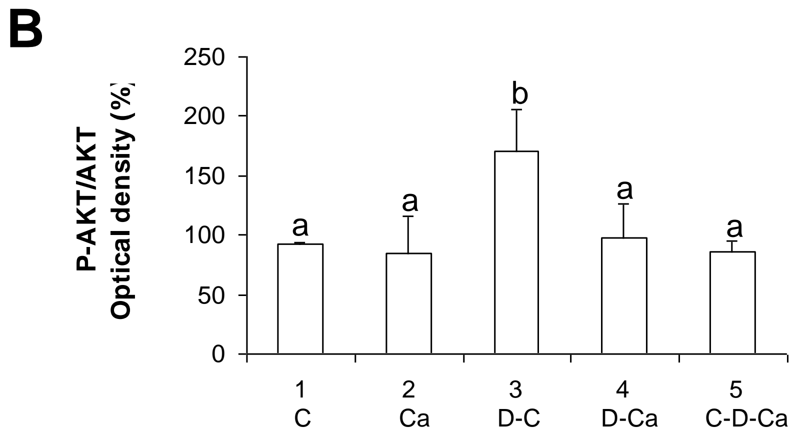


708 **Figure 4.**
709





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712 **Figure 5.**
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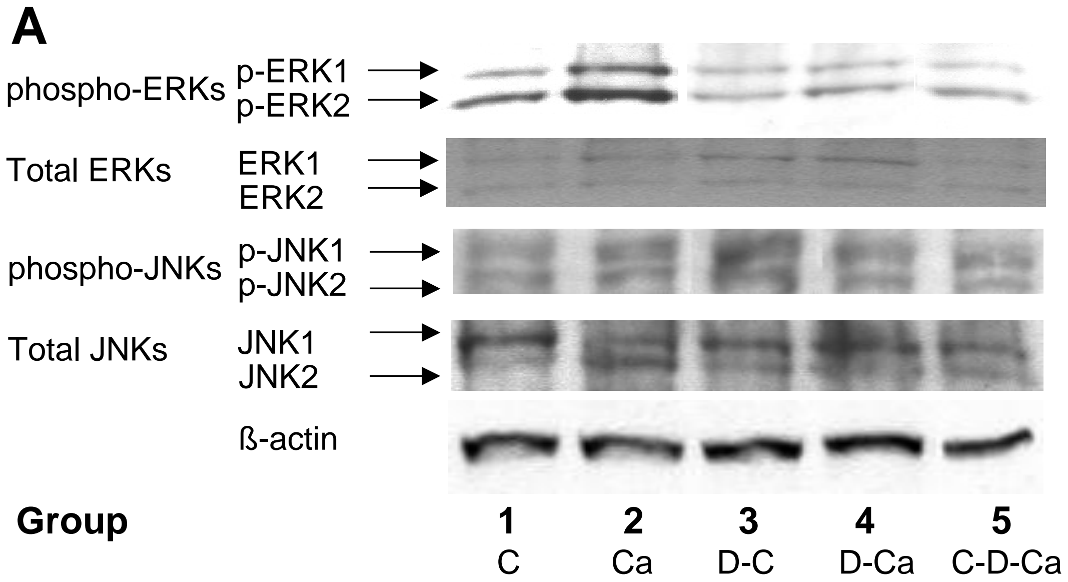

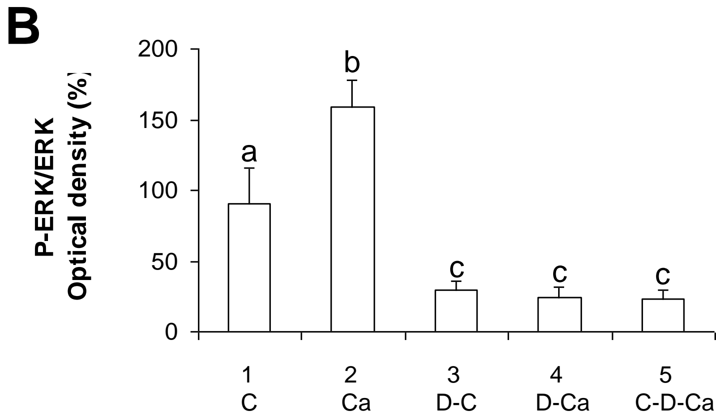

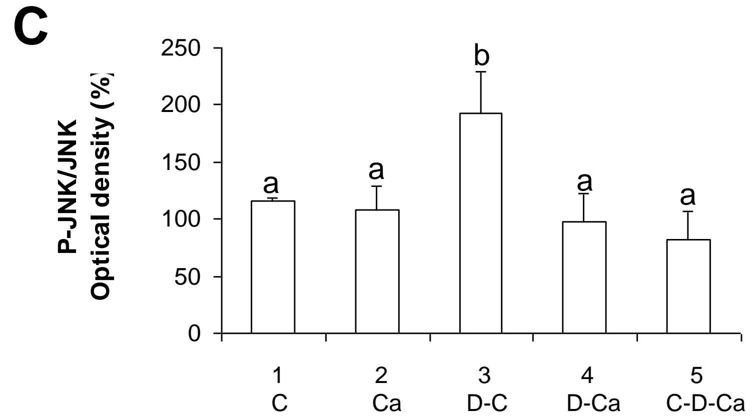

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