COCOA INTAKE ATTENUATES OXIDATIVE STRESS ASSOCIATED WITH RAT ADJUVANT ARTHRITIS

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ABSTRACT

Cocoa contains flavonoids with antioxidant properties. The aim of this study was to ascertain the effect of cocoa intake on oxidative stress associated with a model of chronic inflammation such as adjuvant arthritis. Female Wistar rats were fed with a 5 or 10% cocoa-enriched diet or were given p.o. a quercetin suspension every other day for 10 days. Arthritis was induced by a heat-killed *Mycobacterium butyricum* suspension. Reactive oxygen species (ROS) produced by macrophages, and splenic superoxide dismutase (total, cytoplasmic and mitochondrial) and catalase activities were determined. Clinically, joint swelling in arthritic rats was not reduced by antioxidants; however, the 5% cocoa diet and quercetin administration reduced ROS production. Moreover, the 5% cocoa diet normalized the activities of superoxide dismutase and catalase. In conclusion, a cocoa diet reduces the oxidative stress associated with a chronic inflammatory pathology, although it was not enough to attenuate joint swelling.

Keywords: cocoa, oxidative stress, adjuvant arthritis

Abbreviations: AA, arthritis reference animals; AIN, American Institute of Nutrition; C5, arthritic animals fed 5% cocoa; C10, arthritic animals fed 10% cocoa; EGTA, ethylene glycol tetraacetic acid; H_2DCF-DA, dichlorofluorescein diacetate; Q, arthritic animals treated p.o. with quercetin; p.o., '*per os*' oral administration; RA, rheumatoid arthritis; REF, healthy reference animals; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumour necrosis factor-α

1. Introduction

Flavonoids are polyphenol antioxidant compounds found in vegetables. These benzo-γ-pyrone derivatives can prevent injury caused by free radicals by neutralizing free radicals, chelating metals (principally Fe^{2+} and Cu^{+}) that enhance reactive oxygen species, inhibiting enzymes, and regulating antioxidant defense [1]. Cocoa (product of *Theobroma cacao*) is a natural flavonoid source, mainly containing flavanols such as epicatechin, catechin and procyanidins, and smaller amounts of other flavonoids such as quercetin (3,3',4',5,7-pentahydroxyflavone) [2]. The antioxidant effects of quercetin and catechin have been extensively studied on a variety of inflammatory processes and immune functions [3]. Moreover, other flavanols present in cocoa, such as epigallocatechin-3-gallate and procyanidin B_2, protect Caco2 cells against oxidative stress by reducing ROS production, preventing caspase-3 activation, and increasing antioxidant enzymes as glutathione peroxidase, glutathione reductase and glutathione-S-transferase [4]. Nevertheless, the antioxidant efficacy of flavonoids *in vivo* is less documented. Previous studies show that cocoa flavonoids reduce the macrophage ability to release inflammatory mediators including ROS and nitric oxide *in vitro* and in healthy animals [5-7]. Moreover, a cocoa diet increases the activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase in healthy rat thymus [8].

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune and inflammatory disease characterized by joint inflammation and synovial hyperplasia, involving infiltration of activated T cells and macrophages. Macrophages possess broad proinflammatory, destructive and remodeling capacities, and contribute considerably to inflammation and joint destruction in RA. Activation of macrophages is not restricted to

72 the synovial compartment, but extends to circulating monocytes and other cells of the
73 mononuclear phagocyte system [9]. It is believed that macrophages act as amplifiers of
74 systemic inflammation and it has been suggested that RA severity may be associated
75 with the degree of systemic activation of monocytes/macrophages [9]. Activated
76 macrophages release inflammatory mediators such as proteolytic enzymes, ROS and
77 reactive nitrogen species (RNS) [10]. Among ROS, the superoxide anion ($O_2^{\bullet -}$)
78 increases the vascular permeability and promotes neutrophil migration and can be
79 converted into hydroxyl radical ($HO^{\bullet}$), becoming more aggressive [11]. The SOD is an
80 endogenous ubiquitous enzyme that catalyzes the $O_2^{\bullet -}$ dismutation into H_2O_2.
81 Subsequently, the endogenous enzyme catalase transforms it to H_2O and O_2. SOD
82 shows three isoforms: the cytoplasmic SOD1, the mitochondrial SOD2, and the
83 extracellular SOD3. SOD1 and SOD3 use copper and zinc as cofactors, and manganese
84 is the cofactor of SOD2 [11].
85
86 Oxidative stress must be important in the pathogenesis of RA since oxidation
87 markers and impaired antioxidant status are found in the plasma and synovial fluid of
88 RA patients [12-13]. Moreover, several studies have pointed out the beneficial effect of
89 antioxidants in RA patients [14-15]. Due to the antioxidant properties demonstrated by
90 cocoa, it could be hypothesized that cocoa intake modulates the oxidative stress
91 provoked by an inflammatory disease. The aim of this study was to ascertain the effect
92 of cocoa-enriched diets on the oxidant and antioxidant status of rats with adjuvant
93 arthritis considering oxidative stress of peritoneal macrophages, applied in several
94 studies as systemic inflammatory cells [16-17], and antioxidant status in a secondary
95 lymphoid tissue very rich in lymphocytes and monocytes, as the spleen. At the same

time, a group treated with the flavonoid quercetin, with recognized antioxidant and anti-inflammatory activities was added [18].

2. Materials and methods

2.1. Animals and diets

Nine-week-old female outbred Wistar rats (Harlan, Barcelona, Spain) were fed a standard diet, formulated according to the American Institute of Nutrition (AIN-93M), or a diet containing 5% or 10% of partially defatted *Natural Forastero* cocoa (Nutrexpa, Barcelona, Spain) with 21.2 mg of total phenols/g (Folin-Ciocalteu method). Five and ten percent cocoa diets were prepared from a basal mix (Harlan), in which the proportion of proteins, carbohydrates, lipids and fiber had been modified in such a way that the addition of 5 or 10% cocoa resulted in a final isoenergetic diet with the same macronutrient composition as the AIN-93M diet (Table 1). The diets began 14 days before the arthritis induction and lasted until the end of the study (six weeks later) (Fig. 1).

Animals were randomly distributed in 5 different experimental groups (11-12 rats/group): REF (healthy reference animals), AA (arthritis reference animals), Q (arthritic animals treated p.o. with quercetin at 200 mg/kg on days 0, 2, 4, 6, 8 and 10 after arthritis induction), C5 (arthritic animals fed 5% cocoa) and C10 (arthritic animals fed 10% cocoa) (Fig. 1). Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona and approved by the Catalonian Government.

2.2. Induction and evaluation of adjuvant arthritis

Arthritis was induced in all the animals with the exception of the REF group. Adjuvant arthritis was induced as in previous studies [19-20] by injecting intradermally

123 a suspension of 0.5 mg of heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI,
124 USA) in 0.1 ml of liquid vaseline into the base of the rat tail. Arthritis was clinically
125 assessed by means of hind-paw volume (water plethysmometer LI 7500 Letica, Spain)
126 and arthritic score. The arthritis score was calculated as the sum of the clinical score of
127 the four paws according to scores established by two observers in a blind manner
128 following the criteria: '0' means no signs of inflammation, '1' means erythema or slight
129 swelling in the paw articulations, '2' means erythema and moderate swelling in the paw
130 articulations, '3' means erythema and deep swelling in the paw articulations, '4' means
131 erythema and severe swelling and immobility.
132
133 Body weight, hind-paw volume and arthritis score were determined weekly in all
134 the studied groups. On day 28 post-induction, animals were anaesthetized by an
135 intramuscular injection of ketamine (90 mg/kg; Merial, Lyon, France) and xylazine (10
136 mg/kg; Bayer HealthCare, Kiel, Germany), and peritoneal macrophages, as immune
137 cells involved in the systemic inflammation, and the spleen, as immune cell storage and
138 secondary lymphoid tissue, were obtained. Spleen fragments were immediately frozen
139 at -80 ºC until analysis.
140
141 *2.3. Peritoneal macrophages isolation and ROS production*
142 Peritoneal macrophages were obtained by injecting 40 mL of ice-cold sterile
143 PBS (pH 7.2) into the peritoneal cavity. After 2 min of abdominal massages, cell
144 suspension was aspirated. Macrophages were plated and allowed to attach overnight,
145 then they were washed with warm RPMI medium without phenol red (Sigma-Aldrich)
146 containing 100 IU/mL streptomycin-penicillin. Macrophages were incubated with 20
147 µmol/L of reduced 2',7'-dichlorofluorescein diacetate probe (H_2DCF-DA; Invitrogen,

148 Paisley, UK) for 30 min at 37 ºC. Macrophage ROS oxidized H_2DCF to a fluorescent
149 compound (DCF). Fluorescence was measured at 30, 60 and 90 min by a fluorometer
150 (excitation 538 nm, emission 485 nm).
151
152 *2.4. Spleen superoxide dismutase*
153 A fragment of spleen was homogenized in cold 0.1 g/mL HEPES buffer (20 mM
154 pH 7.2, containing 1 mM ethylene glycol tetraacetic acid EGTA, 210 mM mannitol and
155 70 mM sucrose) and centrifuged (1,500 *g*, 5 min, 4 ºC). The total SOD activity was
156 determined in the supernatant using superoxide dismutase assay kit II (Merck KGaA,
157 Darmstadt, Germany) following the manufacturer's instructions. To determine SOD
158 subtypes, homogenate supernatant was centrifuged at 10,000 *g*. The supernatant and the
159 pellet were used to evaluate the cytoplasmic and mitochondrial SOD activities,
160 respectively. In the mitochondrial SOD quantification, the pellet was resuspended in
161 HEPES buffer, and potassium cyanide (3 mM, Sigma-Aldrich) was added to the
162 samples to inhibit cytoplasmic SOD.
163
164 One unit of SOD is defined as the amount of enzyme needed to exhibit 50%
165 dismutation of the superoxide radical. SOD activity was expressed as units of SOD per
166 g of protein from spleen homogenates (quantified following the Bradford method using
167 the Bio-Rad Protein Assay of Bio-Rad Laboratories Inc., Hercules, CA).
168
169 *2.5. Spleen catalase activity*
170 A spleen fragment was homogenized on ice-cold phosphate buffer (0.1 g/mL; 50
171 mM K_2HPO_4, 50 mM KH_2PO_4 and 1 mM EDTA, pH 7.0) and centrifuged (10,000 g,
172 15 min, 4 ºC). The catalase activity was determined in the supernatant by using a

catalase assay kit (Merck) following the manufacturer's instructions. The detection method was based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2, and the formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen. Catalase activity was expressed as μmol of formaldehyde per min per g of protein from spleen homogenates.

2.6. Statistics

The software package PASW Statistics 18.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. Levene's and Kolmogorov-Smirnov's tests were applied to assay variance equality and normal distribution of the studied groups, respectively. The one-way analysis of variance (ANOVA) followed by Scheffé's post hoc significance test was applied when the assumptions of normality and equal variance were met. However, non-parametric tests (Kruskal-Wallis and Mann-Whitney U) were used to assay significance. Chi squared test was applied in the frequency study. Significant differences were accepted when $P < 0.05$ and were labeled in tables and figures with a different letter.

3. Results and discussion

Rheumatoid arthritis, as well as experimental models of arthritis, have been associated with oxidative stress [12-13]. Therefore, foods with antioxidant properties could help in the treatment of RA decreasing this harmful state [21]. The results obtained here show the effects of cocoa-enriched diets and quercetin treatment on some markers of oxidative stress in adjuvant arthritis after 4 weeks of induction.

The inflammatory status of adjuvant arthritis was measured by means of hind-paw volume, arthritis score and body weight increase. As can be seen in Table 2, maximum hind-paw volume in the AA group was achieved 3 weeks after induction and thereafter tended to reduce. Similar results were observed when considering arthritis score. Quercetin group, which was includes as a positive control, did not prevent arthritis as we expected and even induced a more precocious appearance of arthritis (measured by hind-paw volume or arthritis score, Table 2). These results do not agree with those of Mamani-Matsuda and coworkers [18] who show the curative effect of quercetin (30 mg every 2 days from day 10 after induction) in adjuvant arthritis induced in 6 week-old Lewis rats. The differences could be attributed to events that would change susceptibility to this flavonoid, such as the rat strain (Wistar vs Lewis), age of animals (9-week *vs* 6-week old animals), the protocol (preventive *vs* curative) or even the dose (about 40 mg per rat *vs* 30 mg). Similarly, cocoa diets (groups C5 and C10) were not able to prevent joint swelling, which actually was already detected at day 7 post-induction. However, after maximum paw swelling on day 21 after induction, the animals from groups C5 and C10 underwent a faster recuperation than the AA rats. These results correlate with the body weight increase: during the first two weeks, body weight decreased in all groups receiving arthritis induction, thereafter body weight rose

215 and this recovery was faster in the C5 and C10 groups than in the AA and Q groups
216 (Table 2).
217
218 Macrophages, obtained 4 weeks after arthritis induction, allow the potential
219 oxidative stress in adjuvant arthritis to be estimated (Fig. 2). Macrophages from the AA
220 animals synthesized higher amounts of ROS than those from the REF group
221 ($P < 0.001$). Macrophages from the C5 and Q groups, but not from the C10 group,
222 produced lower ROS than the AA rats ($P < 0.05$). These results indicate the attenuation
223 of the oxidative stress by certain doses of cocoa flavonoids. Similarly, the increased
224 ROS synthesis in AA rats and their modulation by antioxidant compounds has been
225 reported [22-24]. Moreover, the attenuation of ROS by a cocoa diet is in line with
226 results derived from macrophages obtained from cocoa-fed rats, both healthy ones [6]
227 and those with collagen-induced arthritis [7]. Likewise, administration of quercetin was
228 able to decrease ROS concentration in a kidney inflammation model [25]. The
229 inhibition of ROS synthesis by flavonoids can be associated with their free-radical
230 quenching activity [26]. However, it should be noted that the richest cocoa diet did not
231 produce antioxidant effects. This result does not agree with *in vitro* studies showing a
232 dose-dependent antioxidant effect of cocoa [5, 27], which could be due to the different
233 compounds reaching cells. Thus, the *in vitro* antioxidant activity could be partially due
234 to large flavonoid polymers found in cocoa [1], but these polymers are not intestinally
235 absorbed [28]. On the other hand, flavonoids can act as pro-oxidants when they are in
236 high concentration *in vivo* [1, 29]. Previous studies have shown that low concentrations
237 of quercetin inhibit lipid peroxidation in rat liver and protect human leucocytes against
238 oxidative DNA damage, but high concentrations of this flavonoid enhance the hydroxyl
239 radical formation and increase the DNA damage [1].

240

241 In order to protect tissues from oxidative injuries, the body possesses enzymatic
242 antioxidant enzymatic systems such as superoxide dismutases and catalase enzymes. It
243 has been reported that AA decreases serum or synovial SOD and catalase activities
244 together with other endogenous antioxidant systems [22-24, 30]. Here we found that, 4
245 weeks after induction, arthritis produced a decrease in splenic catalase activity
246 ($P < 0.05$; Fig. 3) and, paradoxically, an increase in splenic total and mitochondrial
247 SOD ($P < 0.05$; Fig. 4). The decreased catalase activity could be associated with the
248 consumption of catalase in neutralizing the H_2O_2. On the other hand, increased splenic
249 SOD activities could reflect the response of the body to increased ROS concentrations
250 and/or it could be due to the fact that arthritis was in its recovery phase one month after
251 its induction. Moreover, SOD increase could also be explained by the increase in the
252 oxidative stress found in arthritic rats (Fig. 2), and by the increased tumour necrosis
253 factor-α (TNF-α) secretion present in arthritis [7]. Both oxidative stress and TNF-α are
254 shown to induce SOD synthesis [31-32]. It should be added that a similar increase in
255 SOD activity was found in the plasma of RA patients [33] and in the synovial
256 membrane of mice with collagen-induced arthritis [34].

257

258 Interestingly, the changes in the enzymatic antioxidant systems produced by
259 adjuvant arthritis were totally prevented in the C5 group which was fed with a diet
260 containing 5% cocoa ($P < 0.05$; Fig. 3 and 4). SOD and catalase activities in the C10
261 and Q groups were not significantly different from the AA rats (Fig. 3 and 4). The
262 beneficial effect of a 5% cocoa diet on antioxidant systems in adjuvant arthritis is in line
263 with the effect of other flavonoids or compounds of a botanical origin on this
264 experimental inflammatory model [24, 30], although these studies found a clinical

265 improvement on arthritis. The reduction of oxidative stress by a 5% cocoa diet (Fig. 2),
266 as well as the decrease in TNF-α secretion produced by a similar diet as reported in a
267 previous study [7] could be responsible for the reduction in splenic SOD. The effect of
268 5% cocoa diet on SOD seems to be due to its activity on the mitochondrial SOD
269 (Fig. 4C), a unique and essential isoform for life [35]. On the other hand, cocoa intake
270 normalized the catalase activity (Fig. 3), which is a cytosolic enzyme absent in the
271 mitochondria of most cells [36]. These results suggest that the antioxidant effect of
272 cocoa is exerted in both cellular compartments.
273
274 From the results obtained in this study, it can be concluded that cocoa intake
275 reduces the oxidative stress in macrophages provoked by adjuvant arthritis. This
276 antioxidant effect of cocoa is dependent on the doses employed. Nevertheless, we still
277 need to discover the antioxidant effect of cocoa on joint tissues and to ascertain the
278 relationship between this antioxidant effect and inflammatory markers. Further studies
279 are needed to establish more deeply the role of cocoa, a source of antioxidant
280 flavonoids, as a coadjuvant in the treatment of chronic inflammatory diseases.
281
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Figure legends

Fig. 1. Diagram of the experimental design that began 14 days before arthritis induction and finished on day 28 post-induction. White bars mean the standard diet (Ref, AA and Q groups), pale grey color represents the 5% cocoa diet (C5 group), and dark grey color represents the 10% cocoa diet (C10 group). Striped bars are representative of period after arthritis induction. Animals from Q group were treated with quercetin p.o. on days 0, 2, 4, 6, 8, 10 post-induction (squared bar). At the end of the study, peritoneal macrophages and spleen were obtained from all animals.

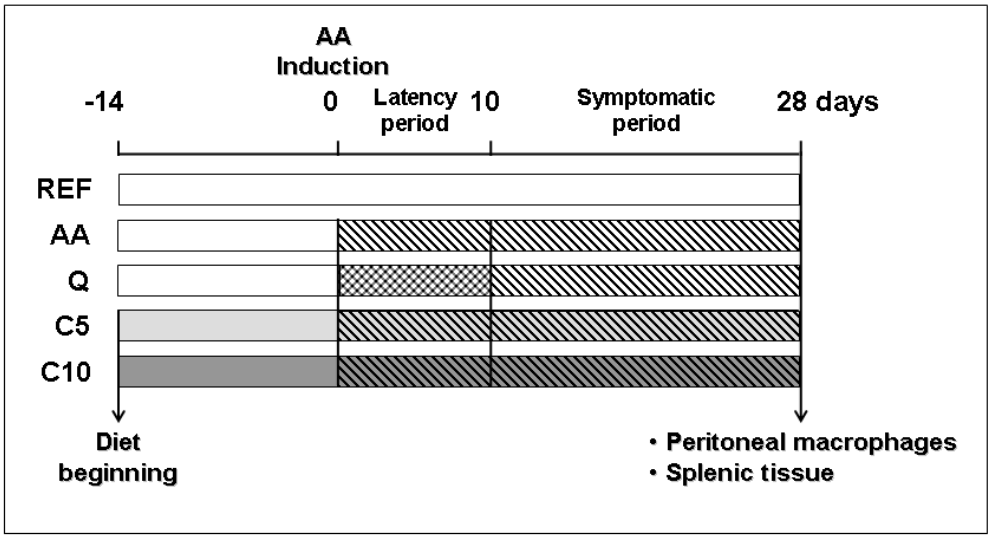


Fig. 2. Time course of ROS production by macrophages, expressed in fluorescence units (FU) and determined by DCF assay. Values are expressed as mean ± S.E.M. (n = 8). Means with different letter (a, b, c) differ, $P < 0.05$ (Kruskal-Wallis and Mann-Whitney U tests).

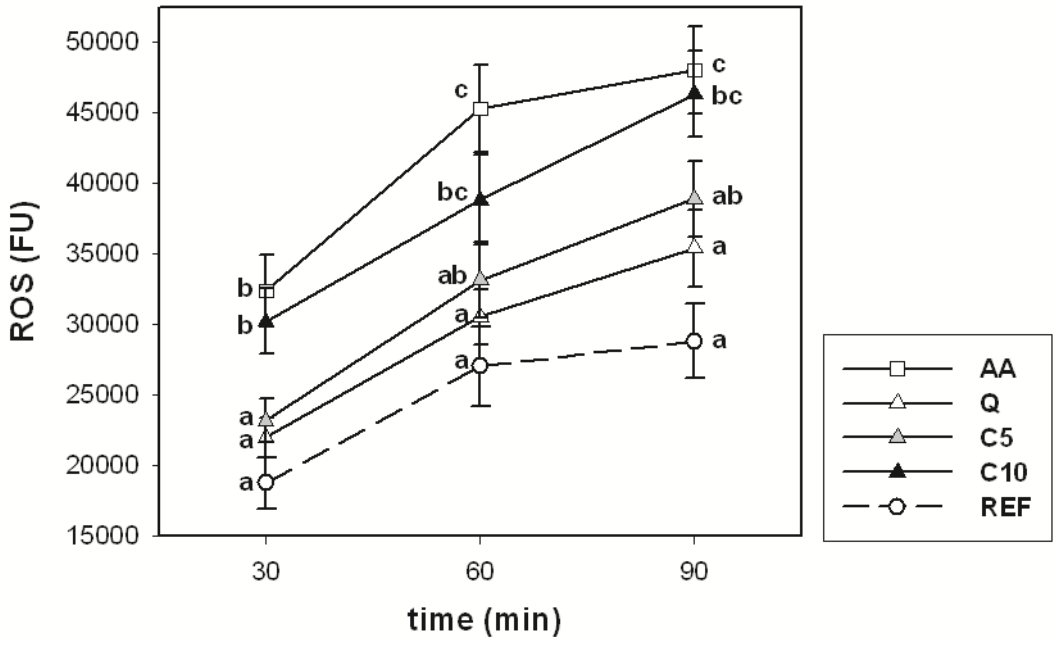


Fig. 3. Catalase activity in spleen homogenates expressed as µmol/min/g of protein. Each bar represents the mean ± S.E.M. (*n* = 11-12). Means with different letter (a, b) differ, P < 0.05 (ANOVA followed by Scheffé's test).

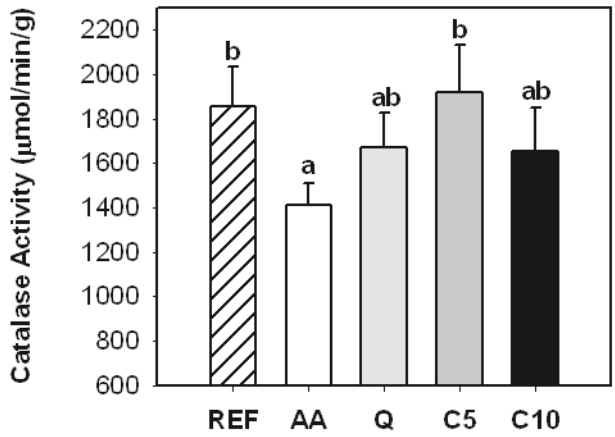
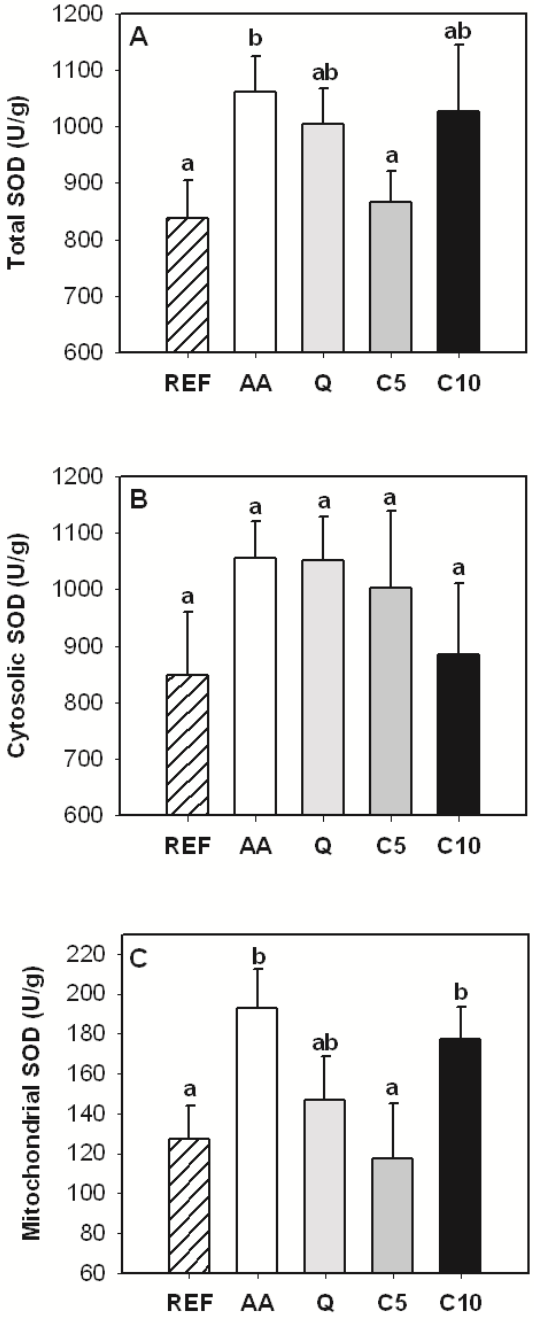


Fig. 4. Activity of SOD subtypes in spleen homogenates: (A) total, (B) cytosolic, and (C) mitochondrial SOD activities. SOD is expressed as units (U)/g of protein. Each bar represents the mean ± S.E.M. (*n* = 11-12). Means with different letter (a, b) differ, P < 0.05 (Kruskal-Wallis and Mann-Whitney U tests).



Tables

Table 1. Composition of the experimental diets (g/kg).

Components	Standard diet (AIN-93M, g/kg)	5% Cocoa diet (g/kg)	10% Cocoa diet (g/kg)
Casein	140	129	118
L-Cystine	1.8	1.8	1.8
Corn starch	465.69	457.69	449.69
Maltodextrin	155	155	155
Sucrose	100	100	100
Soybean oil	40	34.5	29
Cellulose	50	37.25	24.5
Mineral mix (TD94046)	35	35	35
Vitamin mix (TD94047)	10	10	10
Choline bitartrate	2.5	2.5	2.5
tert-Butylhydroquinone	0.008	0.008	0.008
Natural cocoa powder	-	50	100
Cocoa macronutrients:			
Protein		*11*	*22*
Carbohydrate		*8*	*16*
Lipid		*5.5*	*11*
Insoluble fiber		*12.75*	*25.5*
Total cocoa polyphenols [(1)]		1.06	2.12
Cocoa flavonoids [(2)]:			
Epicatechin		*0.10995*	*0.2199*
Procyanidin B_2		*0.08375*	*0.1675*
Catechin		*0.0368*	*0.0736*
Isoquercetin		*0.00275*	*0.0055*
Quercetin		*0.0015*	*0.003*

[1]Cocoa polyphenols were determined by Folin-Ciocalteu method.
[2]Main cocoa flavonoids were determined by HPLC.

Table 2. Effect of flavonoid intake on total body weight ~~increase~~ and arthritis severity (n = 11-12). Body weight (g) and hind-paw volume (mL) values are summarized as mean ± S.E.M. Means or frequencies with different letter (a, b, c) differ, $P < 0.05$ (ANOVA followed by Scheffé's test). Arthritis score is presented as frequency of each score. Frequencies without a common letter differ, $P < 0.05$ (Chi square).

			post-induction day			
	Group		7	14	21	28
Body Weight	REF		211.7 ± 2.6 [a]	220.1 ± 3.1 [a]	227.1 ± 3.4 [a]	231.8 ± 3.6 [a]
	AA		195.6 ± 4.1 [b]	194.8 ± 4.8 [b]	193.8 ± 5.2 [b]	200.2 ± 5.4 [b]
	Q		192.2 ± 3.1 [b]	189.9 ± 2.7 [b]	195.1 ± 3.5 [b]	202.2 ± 3.7 [b]
	C5		185.0 ± 2.5 [c]	183.1 ± 2.6 [c]	189.5 ± 2.5 [b]	198.9 ± 2.4 [b]
	C10		180.0 ± 2.4 [c]	181.3 ± 2.3 [c]	184.2 ± 3.4 [b]	192.9 ± 4.0 [b]
Hind-paw volume [(1)]	REF		0.81 ± 0.01 [a]	0.83 ± 0.01 [a]	0.84 ± 0.01 [a]	0.85 ± 0.01 [a]
	AA		0.82 ± 0.01 [a]	1.27 ± 0.09 [b]	1.74 ± 0.13 [b]	1.43 ± 0.09 [b]
	Q		0.93 ± 0.03 [b]	1.34 ± 0.16 [b]	2.14 ± 0.23 [b]	1.58 ± 0.18 [b]
	C5		0.87 ± 0.02 [b]	1.40 ± 0.11 [b]	1.67 ± 0.12 [b]	1.27 ± 0.09 [b]
	C10		0.86 ± 0.02 [ab]	1.31 ± 0.12 [b]	1.73 ± 0.16 [b]	1.27 ± 0.10 [b]
		Arthritis score range				
Arthritis Score [(2)]	REF	0	11 [a]	11 [a]	11 [a]	11 [a]
		1-4	0	0	0	0
		5-8	0	0	0	0
		9-12	0	0	0	0
		13-16	0	0	0	0
	AA	0	11 [a]	2 [b]	1 [b]	1 [b]
		1-4	1	2	3	5
		5-8	0	8	4	3
		9-12	0	0	4	3
		13-16	0	0	0	0
	Q	0	5 [b]	0 [b]	1 [b]	1 [b]
		1-4	6	5	3	5
		5-8	0	4	3	4
		9-12	0	2	4	1
		13-16	0	0	0	0
	C5	0	5 [b]	0 [b]	0 [b]	0 [b]
		1-4	7	6	3	8
		5-8	0	5	7	3
		9-12	0	1	2	1
		13-16	0	0	0	0
	C10	0	7 [ab]	0 [b]	1 [b]	3 [b]
		1-4	4	6	2	3
		5-8	1	5	6	5
		9-12	0	1	3	1
		13-16	0	0	0	0

[1] Hind-paw volume is shown as the average of the two hind-paw volumes, expressed as mL.

[2] Arthritis score was established according to: '0' means no signs of inflammation, '1' means erythema or slight swelling in the paw articulations, '2' means erythema and moderate swelling in the paw articulations, '3' means erythema and deep swelling in the paw articulations, '4' means erythema and severe swelling and immobility.