

Cocoa-Enriched Diet Enhances Antioxidant Enzyme Activity and Modulates Lymphocyte Composition in Thymus from Young Rats

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Cocoa is a rich source of flavonoids, mainly (–)-epicatechin, (+)-catechin, and procyanidins. This article reports the effect of continuous cocoa intake on antioxidant capacity in plasma and tissues, including lymphoid organs and liver, from young rats. Weaned Wistar rats received natural cocoa (4% or 10% food intake) for three weeks, corresponding to their infancy. Flavonoid absorption was confirmed through the quantification of epicatechin metabolites in urine. Total antioxidant capacity (TAC) and the activity of antioxidant enzymes, superoxide dismutase (SOD) and catalase, were examined. Cocoa intake enhanced TAC in all tissues especially in thymus. Moreover, thymus SOD and catalase activities were also dose-dependently increased by cocoa. It was also analyzed whether the enhanced antioxidant system in thymus could influence its cellular composition. An increase in the percentage of thymocytes in advanced development stage was found. In summary, cocoa diet enhances thymus antioxidant defenses and influences thymocyte differentiation.

KEYWORDS: Catalase activity; SOD activity; cocoa; lymphocyte phenotype; thymocyte differentiation

INTRODUCTION

The production of reactive oxygen species (ROS), including free radicals, is a well-established physiological process which is controlled by intrinsic antioxidant systems. The antioxidant defense comprises enzymatic and nonenzymatic mechanisms. Antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, constitute a first line defense by catalyzing the conversion of specific ROS (superoxide anion (O₂^{•-}) and H₂O₂, respectively) into less reactive or nonreactive products. Non-enzymatic antioxidants comprise an array of molecules, including albumin, glutathione, and uric acid among others, which act neutralizing nonspecifically highly reactive oxidants, such as hydroxyl radical (HO[•]), overproduced when specific intracellular defenses are overwhelmed (1). In certain situations, the intrinsic oxidant/antioxidant balance is shifted toward the production of reactive species and consequently oxidative stress is enhanced. An elevated oxidized state within a cell can be extremely harmful, resulting in radical generation that leads to lipid peroxidation, DNA cross-linking, and the formation of disulfide bonds in proteins (2). Oxidative stress is clearly involved in a wide range of pathological and physiological

disorders such as cancer, cardiovascular disease, and aging (3, 4). Therefore, improving antioxidant defenses could be a key strategy to prevent or reduce the risk of disease.

A great interest in developing functional foods has recently emerged to improve the state of health and well-being, or reduce disease risks (5). Cocoa is a rich source of antioxidants, mainly flavonoids such as (–)-epicatechin, (+)-catechin, and polymers derived from these monomers called procyanidins (6, 7). In this regard, a serving size of certain cocoa-derived products provides more phenolic antioxidants than beverages and fruits such as tea and blueberries, traditionally considered high in antioxidants (8). In many countries, cocoa-derived products are very commonly consumed and in the European Union and US cocoa intake is estimated at ~2.6 and ~2.35 g/day of cocoa powder per capita, respectively (8). Besides considering cocoa as a dietary antioxidant source, it could also be viewed as a natural product with therapeutic properties.

In vitro studies have demonstrated the protective effect of cocoa and its flavonoids on different cellular models of oxidative stress (9–11). However, these effects cannot be directly extrapolated to humans given that bioavailability and metabolism should be taken into account. Cocoa flavonoids are stable during gastric transit (12), rapidly absorbed, and found in plasma after cocoa beverage consumption (13). Flavonoids are extensively metabolized to mainly glucuronides, sulfates, and O-methylated forms during transfer through the small intestine and then again

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in the liver. Nonabsorbed flavonoids reach the colon where gut microflora convert them into phenolic acids which are able to be absorbed and further metabolized in the liver (14). Therefore, flavonoids tested in *in vitro* studies are obviously not the same as the ones that reach cells in *in vivo* conditions.

Some studies have shown that cocoa also presents antioxidant properties *in vivo*. Cocoa intake enhances antioxidant capacity and decreases lipid oxidation products in plasma from healthy humans (15–17) and rats (13, 18). Moreover, cocoa consumption also reduces lipid peroxidation in plasma from individuals with increased oxidative stress (19, 20). Most of those studies were conducted in adult animals or humans after receiving a single cocoa dose. To date, the effects of continuous cocoa consumption on young rats whose immune system is still in maturation (21) remain to be explored.

In this article, we determined the antioxidant status in healthy young rats that were provided a continuous cocoa diet from weaning and during three weeks. The intestinal absorption and metabolism of cocoa flavonoids were assessed by quantifying their metabolites in urine. Then, we focused on the analysis of total antioxidant capacity (TAC) in plasma, lymphoid tissues (spleen and thymus), and liver. SOD and catalase activities in tissues were also evaluated as representative antioxidant enzymatic systems. Given the importance of redox status in lymphocyte maturation and the high antioxidant enzyme activity detected in the thymus, further assays were designed to establish changes in its cellular phenotype.

MATERIALS AND METHODS

Apparatus: LC–MS/MS. High-performance liquid chromatography (HPLC) was performed using a Perkin-Elmer series 200 (Norwalk, CT, USA) equipped with a quaternary pump, a refrigerated autosampler, and a diode-array detector. A triple quadrupole mass spectrometer API 3000 (Applied Biosystems, PE Scienc, Concord, Ontario, Canada) equipped with a Turbo IonSpray source operated in the negative-ion mode was used to obtain the MS and MS/MS data.

Reagents, Standards, and Diets. Reagents and standards were obtained from the following sources: methanol and acetonitrile (HPLC grade) from Scharlau (Barcelona, Spain); formic acid, (–)-epicatechin, (+)-catechin, quercetin, creatinine, β -glucuronidase and sulfatase (type H-2), 2-2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diamonium salt (ABTS), and bovine serum albumin (BSA) from Sigma-Aldrich-Fluka (St. Louis, MO); Trolox from Calbiochem (Darmstadt, Germany); taxifolin and procyanidin B2 from Extrasynthese (Genay, France); and isoquercetin from Promochem (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from Millipore System (Bedford, MA). RPMI 1640 media and fetal bovine serum (FBS) were purchased from PAA (Pasing, Austria). 2-Mercapto-ethanol (ME) was provided by Merck (Darmstadt, Germany).

Fluorescein isothiocyanate (FITC)-conjugated anti-rat CD3 (1F4) MAb, phycoerythrin (PE)-conjugated anti-rat TCR $\alpha\beta$ (R73) and CD4 (OX-35) MAb, and peridinin-chlorophyll-a protein (PerCP)-conjugated anti-rat CD8 α (OX-8) MAb were purchased from BD Biosciences (Heidelberg, Germany). FITC-conjugated anti-rat CD90 (Thy-1) MAb was obtained from Caltag (Burlingame, CA).

Natural Forastero cocoa (Nutrexpa, Barcelona, Spain) was used for this study. The AIN-93G formulation (22), which provides the nutrients required for optimal rat growth, was used as the control diet. The 10% cocoa diet was produced from modified AIN-93G containing 100 g of cocoa per kg. Diet compositions are detailed in **Table 1**.

Phenolic Content of Cocoa and Chow. The total phenolic content was determined by the Folin–Ciocalteu method and was expressed as (+)-catechin equivalents in mg/g (23). The determination and quantification of individual phenolic compounds in cocoa powder and chow (mg/g) were analyzed by HPLC as previously described (24).

Animals and Experimental Design. Dams with 15-day-old Wistar rat litters (50% male, 50% female) were obtained from Harlan

Table 1. Composition of the Experimental Diets (g/kg)^a

components	control chow (AIN-93G)	10% cocoa-enriched chow
casein	200	178
L-cystine	3	3
corn starch	397.486	381.486
maltodextrin	132	132
sucrose	100	100
soybean oil	70	59
cellulose	50	24.5
mineral mix (TD94046)	35	35
vitamin mix (TD94047)	10	10
choline bitartrate	2.5	2.5
TBHQ	0.014	0.014
natural cocoa powder		100
22% protein		
16% carbohydrate		
11% lipid		
25.5% cellulose		
total calories (kcal/kg diet)	3700	3700

^a 10% cocoa-enriched diet was prepared from the AIN-93G control diet removing 72.8 g/kg (16 g/kg of corn starch, 11 g/kg of soybean oil, 25.5 g/kg of cellulose, and 22 g/kg of casein) and adding natural cocoa.

(Barcelona, Spain). Rats were housed in cages of 10 pups per lactating mother in controlled conditions of temperature and humidity in a 12:12 light:dark cycle. At day 21, pups were weaned and randomly assigned to the following dietary groups.

4% Cocoa-Enriched Diet Group (4%-Cocoa Group). Animals received daily 4.8 g of cocoa/kg of rat by oral gavage. According to the chow intake per day, this dose corresponded to ~4% (g of cocoa/100 g of chow). Rats were given free access to control chow and water.

4%-Cocoa Control Diet Group. Animals received daily water (cocoa vehicle) by oral gavage. Rats were given free access to control chow and water.

10% Cocoa-Enriched Diet Group (10%-Cocoa Group). Animals were given free access to water and chow containing 10% (w/w) cocoa (**Table 1**).

10%-Cocoa Control Diet Group. Animals were given free access to water and control chow.

Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals, and experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (ref 3131).

Sample Obtaining. After 2 weeks of dietary treatment, rats were maintained in metabolic cages for 24 h to collect urine output. Urine was centrifuged (2000g, 15 min, room temperature (RT)), acidified by adding HCl (0.2 M in urine), and frozen at –80 °C for further flavonoid metabolite analysis. After 3 weeks of cocoa or control diet, 6-week-old rats were anesthetized to obtain blood by cardiac puncture and thymus, spleen, and liver were excised. Organs were rinsed with a phosphate buffered saline (PBS) solution (pH 7.4) and immediately frozen in liquid nitrogen and then stored at –80 °C until analysis. Blood samples were kept on ice and were centrifuged (3000g, 10 min, 4 °C) to obtain plasma which was stored at –80 °C until antioxidant capacity assays.

Urine Extraction Procedure. Urine was thawed at 4 °C and processed as previously described (25) with some modifications in solid-phase extraction (SPE) support. Briefly, 1 mL of urine was loaded onto a Waters Oasis HLB cartridges (60 mg) (Waters, Mildford, MA) that had been preconditioned with 1 mL of methanol and equilibrated with 2 mL of 1.5 mol/L formic acid in water. The cartridge was washed with 2 mL of 1.5 mol/L formic acid and with 2 mL of water/methanol (95:5; v:v). Elution was achieved with 2 mL of methanol with 0.1% formic acid. The eluate was evaporated to dryness and reconstituted with mobile phase to 100 μ L. Percent recovery of spiked control urine was >71% (\pm 5.3%). Urine creatinine was measured by a colorimetric assay using picric acid (26).

Liquid Chromatography/Tandem Mass Spectrometry (HPLC–MS/MS) Analyses. The chromatographic and spectrometric method was performed as previously described (24). Briefly, a Luna C₁₈ column (50 × 2.0 mm i.d., 5 μm) (Phenomenex, Torrance, CA) was used with a gradient elution with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B at 800 μL/min. The MS and MS/MS parameters were as previously described (24). Each sample was measured by HPLC–MS/MS in the multiple reaction monitoring (MRM) mode with the following transitions: (–)-epicatechin (289/245), glucuronidated epicatechin (465/289), sulfated epicatechin (369/289), *O*-methyl epicatechin (303/288), *O*-methyl epicatechin glucuronide (479/289), sulfated *O*-methyl epicatechin (383/289), sulfated epicatechin glucuronide (545/289), sulfated *O*-methyl epicatechin glucuronide (559/289), procyanidin B2 (577/289), and taxifolin (303/285). Peaks were identified with a product ion scan at a cycle time of 1 s.

To confirm the conjugated forms an enzymatic hydrolysis was performed as described previously (27) with some modifications. A 1 mL urine sample was acidified to pH 5 with 50 μL of 0.58 mol/L acetic acid and incubated at 37 °C for 45 min under the presence of an *Helix pomatia* extract containing 1100 U of β-glucuronidase and 42 U of sulfatase (Sigma). After acidification to pH 2 with 40 μL of 6 mol/L HCl, the urine was loaded onto a preconditioned cartridge and treated as described above. The concentration of (–)-epicatechin metabolites was expressed as epicatechin equivalents (25, 28).

Tissue Homogenates. Tissue samples were homogenized in 5 mL of ice-cold buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) per gram of tissue, using a Polytron (Kinematica, Switzerland). Then, samples were centrifuged (10000g, 15 min at 4 °C) and supernatants were stored at –80 °C until use (<1 month).

ABTS Assay. Total antioxidant capacity (TAC) in plasma and tissue samples was determined by the ABTS method described by Re et al. (29) with modifications. This assay is based on the sample ability, by means of its nonenzymatic antioxidants, to reduce ABTS^{•+} (blue-green color), resulting in a decolorization.

ABTS was dissolved in water at 7 mM, and immediately K₂S₂O₈ was added (2.45 mM final concentration) to convert ABTS into ABTS^{•+} radical cation. This solution was kept in continuous agitation and in darkness for 12–16 h before using it to allow ABTS^{•+} generation (blue-green color). Then, ABTS^{•+} was diluted with distilled water at a 1:50 ratio. The assay was performed in 96-well plates. Trolox, a hydrosoluble vitamin E derivative, was used as a standard. Diluted ABTS^{•+} (100 μL) was added to 100 μL of each sample or standard diluted in phosphate buffered saline solution (PBS) pH 7.4. Absorbance at 420 nm was monitored every 60 s throughout 360 s. The percentage of absorbance inhibition was calculated and plotted as a function of time and concentration. Sample values were interpolated into a trolox standard curve (29). Results were expressed as mM trolox for plasma samples and μmol/g for tissue homogenates.

Antioxidant Enzyme Activities. Superoxide dismutase and catalase activities in tissue samples were determined by means of colorimetric methods using commercial kits (Calbiochem, Darmstadt, Germany). Superoxide dismutase activity assay is based on the detection of superoxide radicals generated by xanthine oxidase addition. A tetrazolium salt was used as a chromogen, and absorbance values were interpolated into a SOD standard curve. Results were expressed as U/mg protein. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Catalase activity was established by the sample ability to react with methanol in the presence of H₂O₂. Formaldehyde produced was measured spectrophotometrically by using purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) as chromogen. Absorbance values were interpolated into a formaldehyde standard curve, and results were expressed as nmol of formaldehyde/min/mg of protein.

Protein content was quantified by the Bradford assay (30) with bovine serum albumin as standard.

Thymocyte Isolation. Thymocyte cell suspension was obtained by passing the thymus through a steel mesh (Collector). Cell suspension was incubated on ice to remove tissue debris by sedimentation for 10 min. Thereafter, cells were centrifuged (500g, 5 min, 4 °C) and resuspended with RPMI media containing 10% FBS and 0.05 mM ME

Table 2. Total Phenolic Content (Folin–Ciocalteu) and Polyphenols (HPLC) in Cocoa Powder and Chow Containing 10% Cocoa (mg/g)^a

	cocoa powder	10% cocoa chow
total polyphenol	21.85 ± 1.08	0.90 ± 0.54
(+)-catechin	0.74 ± 0.05	0.10 ± 0.004
procyanidin B2	1.68 ± 0.11	0.23 ± 0.01
(–)-epicatechin	2.20 ± 0.10	0.34 ± 0.01
isoquercetin	0.05 ± 0.001	0.02 ± 0.00
quercetin	0.03 ± 0.00	0.01 ± 0.00

^a Results were expressed as mean ± SD (n = 3).

(complete media). Cell counting and viability were determined by fluorescence light microscopical analysis.

Phenotype by Immunofluorescence Staining and Flow Cytometry Analysis. Thymocytes were stained with anti-rat MAb conjugated to FITC, PE, or PerCP: anti-TCRαβ (R73), anti-CD4 (OX-35), anti-CD8α (OX-8), anti-CD3 (1F4), and anti-CD90 (Thy-1). 2 × 10⁵ cells were labeled with saturating concentrations of FITC-, PE-, and PerCP-MAb in PBS pH 7.2 containing 1% FBS and 0.09% NaN₃ (30 min, 4 °C, in darkness). A negative control staining using an isotype-matched MAb was included for each sample. After washing with PBS pH 7.2, cells were fixed with 0.5% p-formaldehyde and stored at 4 °C in darkness. Analyses were performed using a Coulter Epics XL2 Corporation cytometer (Miami, FL), and data was assessed by cytometer software (Summit V3.1, Cytomation, Inc). Results were expressed as percentage of positive cells in the lymphocyte population previously selected according to their forward (FSC) and side (SSC) scatter characteristics.

Statistical Analysis. Results from figures and tables were expressed as mean ± SEM, except for **Table 2** that was expressed as mean ± SD. Data of epicatechin and metabolites concentrations were skewed (Kolmogorov and Levene tests). The Wilcoxon test for related samples was carried out to compare changes between both cocoa intakes. Conventional one-way ANOVA was performed, considering diet groups as independent variables. When cocoa intake had a significant effect on the dependent variable, Bonferroni's test was applied. Significant differences were accepted when P < 0.05. After comparing the experimental groups, no statistical differences were seen between both control groups. Therefore, in order to simplify the interpretation of the results, the data from the two control groups (gavage and nongavage) were pooled in the graphs. SEM of control groups in graphs indicates the low dispersion for these groups. Significant differences marked in the graphs are owing to the comparison between treatment group and its corresponding control group. All data were analyzed using SPSS Statistical Analysis System, V. 11.5 (SPSS).

RESULTS

Polyphenols Content in Cocoa and Chow. Total and individual phenolic content in cocoa powder and 10% cocoa chow is shown in **Table 2**. Both cocoa powder and 10% cocoa chow contained substantial amounts of polyphenols, whereas the phenolic levels of control diet (AIN-93G) were under the detection limit. Therefore, in our study polyphenols were provided by cocoa.

Epicatechin Metabolites in the 24 h Rat Urine. The quantification of epicatechin metabolites is established as a biomarker of flavonoid absorption (31). The identification of metabolites in biological samples was based on 3 parameters: comparison of retention time of available standard, MRM metabolite transition and epicatechin transition [with higher DP in collision-induced dissociation (CID)/MS/MS conditions], and product ion spectra.

Epicatechin aglycone was identified in urine samples from cocoa fed rats (peak 1) (**Figure 1**). Three epicatechin glucuronides and three glucuronidated *O*-methyl epicatechins were identified in the rat urine (**Figure 1**). Peaks 2, 3, and 4 represent the glucuronidated metabolites with MRM of 465/289 that were

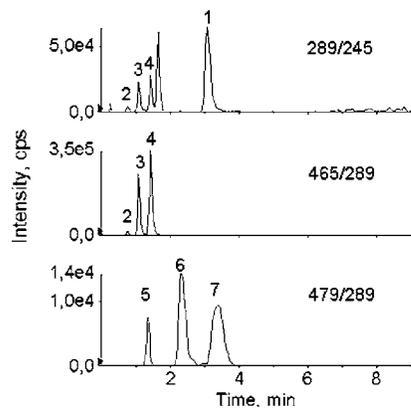


Figure 1. Representative multiple reaction monitoring chromatogram of one rat urine. Peaks: (1) (–)-epicatechin, (2, 3, 4) glucuronided epicatechin, (5, 6, 7) glucuronided *O*-methyl epicatechin.

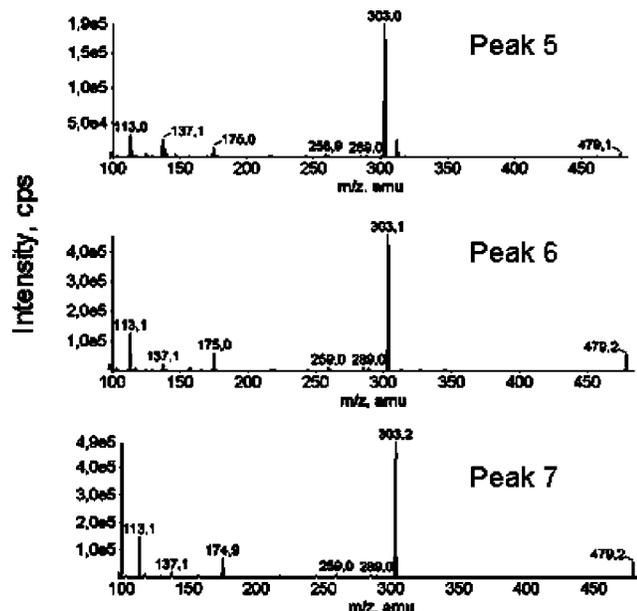


Figure 2. Product ion spectra of peaks 5, 6, and 7. They confirm the three *O*-methyl epicatechin glucuronides in different positions.

confirmed with the epicatechin transition (289/245). Peaks 5, 6, and 7 with MRM of 479/289 correspond to glucuronided *O*-methyl epicatechin identified with the product ion scan of the deprotonated molecule (m/z 479). The product ion spectra of the three peaks showed the deprotonated molecule (m/z 479) and the ions m/z 303 corresponding to methyl epicatechin, m/z 137 corresponding to the classic epicatechin-related A-ring fragment, and m/z 175 and m/z 113 corresponding to the glucuronid acid (**Figure 2**). These confirming results agree with those of Schroeder et al. (28), who showed the spectra of authentic standards of various glucuronided methyl-epicatechin isomers.

The low concentration of other epicatechin metabolites did not allow us to confirm their presence by product ion scan or by MRM transitions (32).

Epicatechin and Epicatechin Metabolites in the Rat Urine.

After 2 weeks of dietary supplementation with cocoa (gavage or chow), epicatechin and epicatechin metabolites were found in the urine of all rats, whereas no metabolites were detected in control rat urines. **Table 3** shows the amount of epicatechin and its metabolites in urine after diets with 4% or 10% cocoa. Cocoa metabolites found in urine were proportional to cocoa intake, but no significant differences were found between both

Table 3. Mean (\pm SEM) of (–)-Epicatechin, Glucuronided Epicatechin, and Glucuronided *O*-Methyl Epicatechin (μ mol/g Creatinine) from Rat Urine Excreted after Administration of 4% Cocoa in Gavage or Chow Containing 10% (w/w) Cocoa ($n = 6$ –12)

	4%-cocoa group	10%-cocoa group
(–)-epicatechin	1.92 \pm 0.93	4.00 \pm 1.85
glucuronided epicatechin	14.26 \pm 1.60	25.57 \pm 18.14
<i>O</i> -methyl epicatechin glucuronide	0.53 \pm 0.07	0.99 \pm 0.62
total epicatechin metabolites	16.66 \pm 2.20	30.55 \pm 18.53

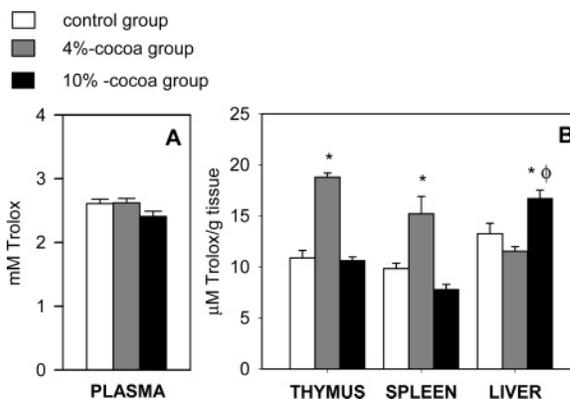


Figure 3. Plasma (A), thymus, spleen, and liver (B) total antioxidant capacity (TAC) in young rats after three weeks of cocoa intake. Each bar represents the mean \pm SEM ($n = 9$ –15). * $P < 0.05$ in 4%- or 10%-cocoa groups vs their respective control groups. $\phi P < 0.05$ in 4%- vs 10%-cocoa group.

Table 4. Percentages of Thymocytes Expressing Low Levels and High Levels of TCR $\alpha\beta$, and the Coreceptors CD4 and/or CD8 on Their Surface in Young Rats after Three Weeks of 10% Cocoa Intake^a

	control group (%)	10%-cocoa group (%)
TCR $\alpha\beta$ ^{high}	13.24 \pm 2.81	13.88 \pm 1.31
TCR $\alpha\beta$ ^{low}	61.94 \pm 2.92	49.16 \pm 3.18 *
total TCR $\alpha\beta$ +	75.18 \pm 2.80	63.05 \pm 4.14 *
CD8+CD4+ (DP)	88.61 \pm 0.22	82.19 \pm 1.20 *
CD8–CD4– (DN)	1.90 \pm 0.09	3.41 \pm 0.19 *
total immature thymocytes (CD8+CD4+, CD8–CD4–)	90.52 \pm 0.22	85.59 \pm 1.07 *
CD8+CD4– (SP)	3.42 \pm 0.59	4.67 \pm 0.76
CD8–CD4+ (SP)	6.07 \pm 0.71	9.75 \pm 0.95 *
total mature thymocytes (CD8+CD4–, CD8–CD4+)	9.18 \pm 0.45	14.41 \pm 1.01*

^a Values represent the mean \pm SEM ($n = 4$). * $P < 0.05$ in 10%-cocoa group vs control group.

groups ($P > 0.05$). A dose-dependent excretion and the high variability among different rats were described by Baba et al. (33).

Effect of Cocoa Diet on Plasma and Tissue Total Antioxidant Capacity (TAC). TAC was measured in plasma, thymus, spleen, and liver by means of ABTS assay (**Figure 3**). Plasma TAC from both cocoa groups did not significantly differ from that of control rats (**Figure 3A**). However, tissue TAC was significantly improved: 4% cocoa diet enhanced thymus and spleen TAC by ~ 73 and 55%, respectively ($P < 0.05$). Unexpectedly, 10% cocoa diet did not affect thymus and spleen

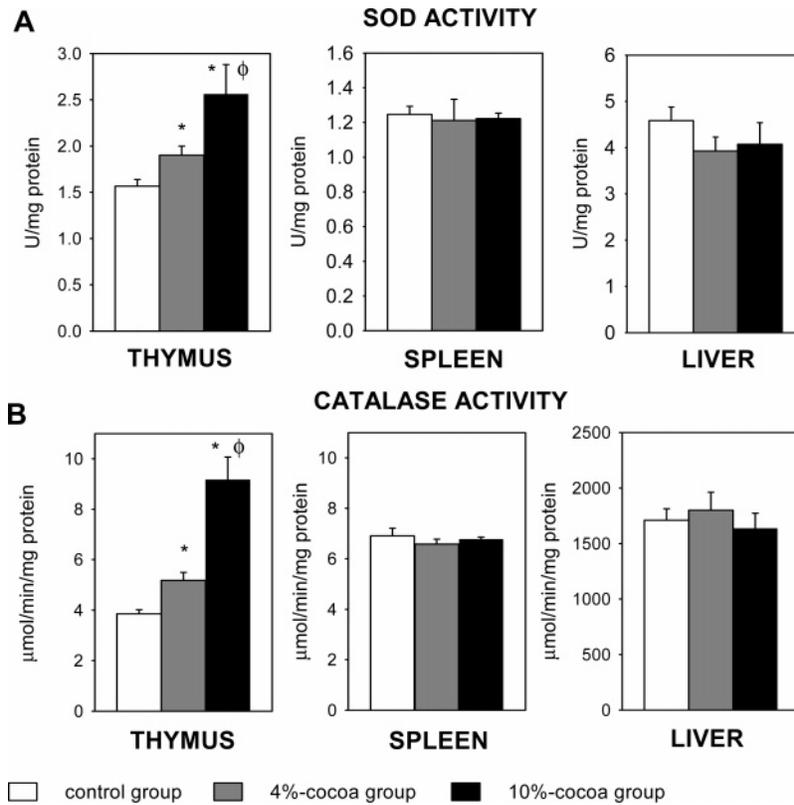


Figure 4. Thymus, spleen and liver superoxide dismutase (SOD) (A) and catalase (B) activities in young rats after three weeks of cocoa intake. Each bar represents the mean \pm SEM ($n = 9-15$). * $P < 0.05$ in 4%- or 10%-cocoa groups vs their respective control groups. $\phi P < 0.05$ in 4%- vs 10%-cocoa group.

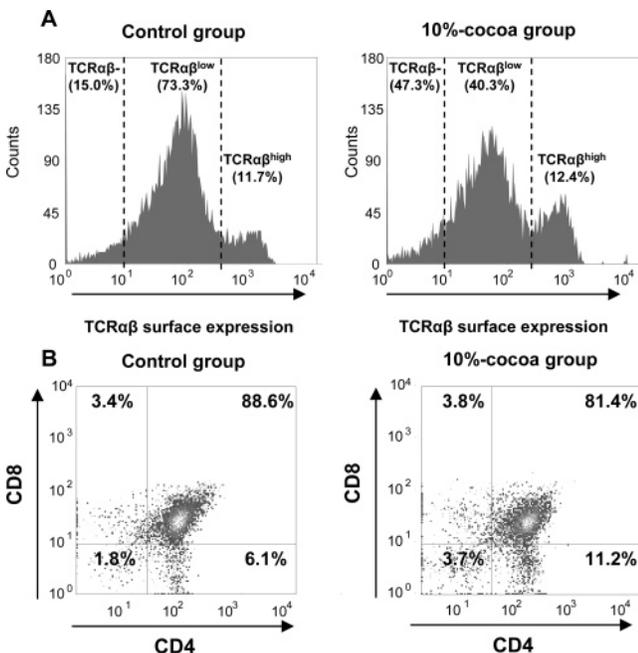


Figure 5. Histograms showing the distribution of thymocytes according to the TCR $\alpha\beta$ surface expression from a representative control rat and a 10% cocoa fed rat (A). Biparametric histograms showing the distribution of thymocytes according to the surface expression of coreceptors CD4 and CD8 from a representative control rat and a 10% cocoa fed rat (B).

TAC, but was capable of increasing liver TAC by $\sim 26\%$ ($P < 0.05$) (Figure 3B).

Effect of Cocoa Diet on Thymus, Spleen, and Liver Antioxidant Enzymes. SOD and catalase activities were measured in thymus, spleen, and liver after 3 weeks of treatment.

Cocoa diets did not modify SOD and catalase activities in spleen and liver, but interestingly, this diet dose-dependently enhanced both enzyme activities in thymus, achieving an increase of 1.64-fold in SOD and 2.4-fold in catalase activities with respect to control groups ($P < 0.05$) (Figure 4).

Effect of Cocoa Diet on Thymocyte Phenotype. Thymocyte phenotype was analyzed in 10% cocoa fed rats and its corresponding control group. In both groups, 99% of thymocytes expressed CD90 and CD3 on their surface. Thymocyte differentiation is characterized by the expression of well-defined cell-surface markers, including CD4 and CD8, as well as T-cell receptor (TCR $\alpha\beta$). Thus, single positive (SP) cells (CD8+CD4- and CD8-CD4+) with high levels of TCR $\alpha\beta$ surface expression (TCR $\alpha\beta$ ^{high}) corresponded to the more mature thymocytes present in the thymus. 10% cocoa diet significantly decreased the proportion of T cells with low expression of TCR $\alpha\beta$ ($P < 0.05$ vs control group); however, the proportion of mature lymphocytes (TCR $\alpha\beta$ ^{high}) was not significantly affected (Table 4 and Figure 5A). Moreover, when coreceptor was studied, a higher percentage of thymocytes in advanced development stage, specifically SP CD4+ cells ($P < 0.05$ vs control group), and a lower proportion of developing DP T cells (CD8+CD4+) were found in 10%-cocoa group ($P < 0.05$ vs control group) (Table 4 and Figure 5B). However, 10% cocoa increased the proportion of double negative cells (DN or CD8-CD4-), which also may act as non-T cell precursors ($P < 0.05$) (Table 4).

DISCUSSION

Previous *in vitro* studies showed the inhibitory effects of cocoa flavonoids on ROS production from activated immune cells (10, 34). This article reports for the first time the effect of continuous cocoa intake on lymphoid tissue antioxidant capacity

in young rats. The cocoa used in this study contained 2.2% (w/w) of total polyphenols, (–)-epicatechin being the major monomeric compound (~10% of total polyphenols). Weaned rats were fed with cocoa-enriched diets for three weeks, which corresponds to their infancy (puberty is estimated at 7–9 weeks in both sexes) (35). Two cocoa dosages were tested: 4% of food intake administered by oral gavage and 10% included in the chow. In both administration protocols, cocoa flavonoids were absorbed and metabolized by rats as demonstrated by the total amount of flavonoid metabolites excreted in urine that is well correlated with flavonoid absorption (31). Although bioavailability may be affected by the type of oral administration (gavage vs chow), our results showed a dose-dependent epicatechin absorption regardless of whether cocoa was given by oral gavage or was included in the diet.

Given that cocoa flavonoids were absorbed, antioxidant capacity was expected to be increased. However, no differences in plasma TAC were found among our groups, and this could be attributed to the short plasmatic half-lives of flavonoids. In this regard, a transitory increase of plasma TAC 10–45 min postgavage, correlating with the plasmatic peak of epicatechin, was detected in rats (13, 18). In our study, blood from 4%-cocoa fed rats was obtained ~20 h postgavage and 10%-cocoa fed rats were deprived of cocoa-enriched chow ~2 h before blood collection. Therefore, the gap between cocoa intake and blood collection could explain the lack of plasma TAC changes among groups.

Although plasma TAC was not modified by cocoa intake, tissue TAC were significantly enhanced. A hierarchy in reducing activity was observed: thymus > spleen > liver. This could be attributed to flavonoid accumulation in specific target tissues allowing a maintained enhancement of their antioxidant capacity. In this regard, it has been described that, after long-term consumption, quercetin, a flavonoid also present in cocoa, was accumulated mainly in lung > testes > kidney > thymus in a dose-dependent manner (34). The effects on thymus and spleen TAC were found not to be significantly dose-dependent. Thus, 4% cocoa diet produced a strong increase in both thymus and spleen TAC, whereas 10% cocoa diet only increased TAC in liver. One possible reason for this fact may be the activation of oxidative pathways in thymus and spleen as a cell compensatory mechanism triggered by high levels of antioxidants accumulated in those tissues (4). On the contrary, in the liver the metabolic rate of flavonoids would decrease their antioxidant capacity and therefore an oxidative compensatory effect would not occur. However, further studies must be conducted to clarify this hypothesis.

Apart from TAC, the activity of endogenous antioxidant enzymes was also analyzed. The cell lacks a specific system to remove hydroxyl radical (HO•), considered the most reactive oxygen species responsible for cell damage. Therefore, the scavenging of O₂^{•-} and H₂O₂ by SOD and catalase enzymes, respectively, is crucial to prevent HO• generation. In the present study, thymus SOD and catalase activities were dose-dependently increased by cocoa intake, whereas spleen and liver were not affected. Cocoa antioxidants, especially flavonoids, seem to be responsible for the enzyme activity enhancement. In this regard, polyphenols can increase SOD and catalase activities by neutralizing their substrates (O₂^{•-} and H₂O₂, respectively) and by upregulating the expression of these enzymes (37). Both SOD and catalase activities increased only in the thymus that possibly due to a higher flavonoid accumulation than in other tissues as previously described (36). The higher amount of free epicatechin, proportional to the cocoa dose ingested, could

reflect the major target concentration of free epicatechin in thymus (lipophilic tissue) in a dose-dependent manner due to its partition coefficient (38, 39).

In addition, spleen and liver contain higher proportions of phagocytes producing ROS than thymus (macrophages in the red pulp of spleen and Kupffer cells in the liver). Therefore, the high activity of SOD and CAT in thymus could be in part due to a lower consumption of such enzymes in this tissue.

The influence of cocoa on thymus antioxidant activity led us to believe that cocoa could also affect lymphocyte composition as we previously found in spleen and gut-associated lymphoid tissue (GALT) (40). This study shows that 10% cocoa diet seems to accelerate the progress of immature thymocytes (DN and DP cells with TCRαβ^{low} expression) toward more mature T cell stages (SP cells with TCRαβ^{high}). Thus, 10% cocoa diet may influence the microarchitecture and cell signaling, inducing changes in T cell development. It has been described that a maintained reducing environment of the cell stimulates a slight shift toward a mildly oxidizing environment that promotes cell differentiation and maturation (4). Therefore, a continuous antioxidant environment in thymus may promote a mild oxidant state that favors lymph maturation. On the other hand, DN cells, a subset whose proportion was also increased by cocoa diet, have multilineage potential, including B cell, T cell, myeloid cell, natural killer cells, and dendritic cells (41). In this sense, high cocoa intake may promote the differentiation of other immune cell subsets. During infancy the immune system is still in development and consequently the body is more susceptible to infection; therefore enhancing lymphocyte differentiation may be beneficial to prevent or reduce the risk of disease.

In summary, cocoa was effectively absorbed and metabolized by young rats after continuous intake, but did not affect plasma antioxidant activity. However, our results show the enhancement of antioxidant capacity in tissues including thymus, spleen, and liver. In the thymus, the richest cocoa diet produced a strong increase in the activity of antioxidant enzymes and may also enhance thymic maturation.

SAFETY

Rat urine samples were considered as potentially infectious. Guidelines for work with organic solvents and acids were respected. Universal precautions for the handling of chemicals and fluids were applied.

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