

Effects of a cocoa diet on an intestinal inflammation model in rats

Teresa Pérez-Berezo¹, Carolina Ramírez-Santana¹, Angels Franch^{1,2}, Sara Ramos-Romero¹, Cristina Castellote^{1,2}, Francisco J Pérez-Cano^{1,2} and Margarida Castell^{1,2}

¹Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona; ²Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), Barcelona E-08028, Spain

Corresponding author: Margarida Castell, Faculty of Pharmacy, Department of Physiology, Av Joan XXIII s/n, Edifici B, 3^a planta, Barcelona 08028, Spain. Email: margaridacastell@ub.edu

Abstract

Cocoa is a rich source of fiber and flavonoids with recognized antioxidant and anti-inflammatory potential. The aim of this study was to evaluate the effects of a cocoa-enriched diet on rats with dextran sulfate sodium (DSS)-induced colitis. Wistar rats were fed with either a 5% cocoa diet or standard diet. Colon inflammation was induced by DSS in the drinking water: 5% for six days and 2% over the following nine days. Colitis was assessed by body weight loss, stool consistency and blood presence in stools. A group of animals fed standard diet was treated with quercitrin (1 mg/kg) after colitis establishment. After two weeks of DSS treatment, the colon oxidative and inflammatory status and lymphocyte composition from blood and mesenteric lymph nodes (MLNs) were assessed. The cocoa-fed group did not exhibit amelioration of clinical colitis but displayed higher antioxidant activity than the colitic reference group by the restoration of colon glutathione content and prevention of lipid peroxidation. The cocoa diet showed anti-inflammatory potential because it down-regulated serum tumor necrosis factor- α , colon inducible nitric oxide synthase activity and decreased colon cell infiltration. The lymphocyte composition in MLNs was not modified by drinking DSS, but there was an increase in the proportion of natural killer and regulatory T-cells in the blood. These changes were not modified by cocoa. In conclusion, cocoa intake may help to inhibit the negative oxidative effects consequent to colitis, although this action is not enough to abrogate the intestinal inflammation significantly.

Keywords: cocoa, flavonoid, dextran sulfate sodium, colitis

Experimental Biology and Medicine 2012; **237**: 1181–1188. DOI: 10.1258/ebm.2012.012083

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) constitute the principal forms of chronic inflammatory bowel disease (IBD). Although the exact etiology of both CD and UC remains unclear, IBD inflammation is thought to result from a complex interaction of genetic and environmental factors that leads to altered immunological responses to gut microbiota.¹

Current treatments for IBD include immunosuppressant or biological drugs blocking key molecules of the inflammatory cascade. However, significant side-effects have been reported, thereby limiting their use.² Because of this, the use of complementary or alternative interventions is becoming widespread and an increasing number of studies show the potential of biologically active nutrients, which may play an important role in the immune response modulation of IBD.³ In this context, since the colon may be subjected to oxidative stress during active inflammation, the

incorporation of dietary antioxidants might help to reduce mucosal inflammation.⁴ Commonly occurring dietary polyphenols, particularly flavonoids, have been reported to possess remarkable antioxidant and anti-inflammatory capabilities in experimental conditions.^{5,6} Cocoa and cocoa-based products are the natural sources of flavan-3-ols such as (–)-epicatechin, (+)-catechin and their oligomers, the procyanidins.⁷ Moreover, cocoa is considered an excellent source of dietary fiber, which retains significant amounts of polyphenolic compounds and plays a key role in their transportation through the gastrointestinal tract.⁸ Cocoa procyanidins seem to reach the colon intact, where they are metabolized by the intestinal microbiota into various phenolic acids.⁹ Several studies have demonstrated the antioxidant capacity of cocoa flavonoids and their metabolites.¹⁰ In addition, cocoa has exhibited *in vitro* and *in vivo* anti-inflammatory and regulatory effects on immune cells involved in innate and acquired immunity.^{11–13}

Table 1 Polyphenol composition of the cocoa used in the study

Compound	mg/g
Procyanidin B ₂	1.487
Catechin	0.740
Epicatechin	1.917
Isoquercetin	0.053
Quercetin	0.020
Total polyphenols	21.85

Individual phenolic compounds and total phenolic content in cocoa powder were determined by high-performance liquid chromatography and the Folin-Ciocalteu method, respectively, as previously described¹⁵

As the oral administration of dextran sulfate sodium (DSS) is a widely used and well-characterized model of colitis in rodents and shares clinical and pathological characteristics with human IBD,¹⁴ the aim of this study was to evaluate the effects of a diet containing cocoa on DSS-induced chronic colitis in rats. In particular, we focused on the response of mesenteric lymph nodes (MLNs) and blood lymphocytes and on the assessment of diverse biochemical variables associated with colonic oxidative stress induced by DSS-induced colitis. In addition, the effects of cocoa were compared with those exerted by quercitrin, a glycoside of the flavonoid quercetin with recognized anti-inflammatory activity in the DSS model⁶ which was used as a control treatment.

Materials and methods

Reagents

DSS of molecular weight 36–50 kDa was obtained from MP Biomedicals (Illkirch, France). RPMI 1640 medium, glutamine, penicillin, streptomycin and fetal bovine serum were purchased from PAA (Pasching, Austria). Quercitrin was obtained from Extrasynthèse (Genay, France). Defatted Natural Forastero cocoa containing 21.85 mg/g polyphenols (Table 1) was provided by Nutrexp SL (Barcelona, Spain). All other reagents, unless otherwise stated, were purchased from Sigma-Aldrich (Madrid, Spain).

Animals and diets

Five-week-old female Wistar rats (Harlan, Barcelona, Spain) were housed in cages under controlled conditions of temperature ($22 \pm 2^\circ\text{C}$) and humidity in a 12:12 light-dark cycle. All experimental procedures were reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (reference DMAH3874).

Animals had free access to water and chow. The AIN-93M formulation¹⁶ was used as the standard diet. A diet containing 50 g/kg cocoa was produced from a modification of the AIN-93M formula as previously described¹² and was provided for three weeks prior to DSS administration and until the end of the study (Figure 1).

Induction and assessment of colitis

Colitis was induced by *ad libitum* administration of 5% DSS (w/v) in drinking water for six days and 2% DSS for the following nine days. Animals were randomly assigned to four experimental groups ($n = 12/\text{group}$; Figure 1): a group receiving a standard diet and water without DSS (REF); a group fed the standard diet and water with DSS (DSS); a group fed the standard diet and water with DSS that was treated daily with quercitrin (1 mg/kg, orally) from the day that the DSS dose was reduced to 2% (DSS-Q); and a group fed the cocoa diet from three weeks prior to DSS drinking and until the end of the study (DSS-C). The dosing protocol for quercitrin was established according to reported studies showing an amelioration of the DSS-induced inflammatory process.⁶

Clinical assessment of colitis included daily evaluation of body weight, stool consistency (normal, loose or diarrhea) and the presence of macroscopic blood in the stools. These variables allowed calculating a disease activity index (DAI) with a 0–4 point severity scale.¹⁷ At the end of the study (Figure 1), animals were anesthetized intramuscularly with ketamine (90 mg/kg) and xylazine (10 mg/kg). Blood, MLNs, small intestine and colon were obtained from each animal.

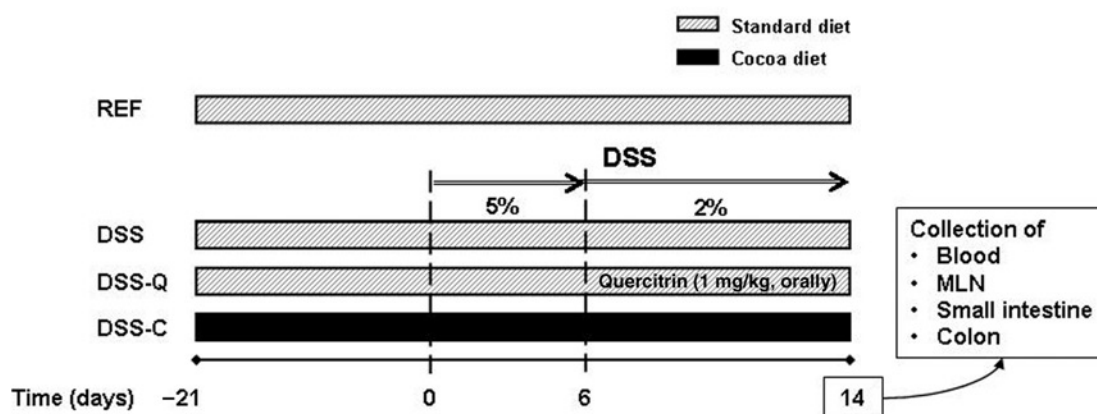


Figure 1 Experimental design: three groups (Reference [REF], dextran sulfate sodium [DSS] and DSS-quercitrin [DSS-Q]) were fed a standard diet, and a group (DSS-C) was fed a cocoa diet throughout the study from three weeks before colitis induction. DSS was incorporated into the drinking water at 5% and 2% over six and nine days, respectively. Quercitrin was administered by oral route (1 mg/kg/day) to the DSS-Q group from day 6

MLNs and blood lymphocyte phenotype

MLNs and blood lymphocytes were isolated as previously described.¹² Lymphocyte phenotype was determined by three-color immunofluorescence staining and flow cytometry analysis. Mouse anti-rat monoclonal antibodies (MAb) used here included: fluorescein isothiocyanate (FITC)-conjugated anti-T-cell-receptor (TCR) $\alpha\beta$ (R73), CD45RA (OX-33), CD4 (OX-35) and CD25 (IL-2R α chain OX-39) MAb; phycoerythrin-conjugated anti-CD4 (OX-35) and NK1.1 (10/78) MAb; peridinin-chlorophyll-protein-conjugated and anti-CD8 α (OX-8) MAb (all from BD Biosciences, Madrid, Spain); and allophycocyanin-conjugated anti-Foxp3 (FJK-16a) MAb (eBioscience, Frankfurt, Germany).

Extracellular and intracellular staining was carried out as in previous studies.¹² Flow cytometry analysis was performed on a Cytomics FC500-MPL cytometer (Beckman Coulter, Miami, FL, USA) and data were analyzed by the software Summit V3.1 (Cytomation, Fort Collins, CO, USA). Lymphocyte subsets were defined as B (CD45RA+), Th (CD4+TCR $\alpha\beta$ +), Tc (TCR $\alpha\beta$ +CD8 α +), activated Th (T_{act}; CD4+CD25+Foxp3-) and regulatory Th (T_{reg}; CD4+CD25+Foxp3+). Results are expressed as a percentage of positive cells in the total lymphocyte population or, in some cases, are shown as a percentage of positive cells in a specific lymphocyte subset (T_{act} and T_{reg} in Th cells).

Blood leukocyte counts

Total white blood cell counts were determined automatically by means of a Coulter Counter JT hemocytometer (Beckman Coulter) calibrated for rat samples. Differential leukocyte counts were microscopically established in blood smears stained using the May-Grünwald-Giemsa technique.

Quantification of tumor necrosis factor- α

Serum tumor necrosis factor (TNF)- α concentration was quantified using a commercial rat cytokine bead immunoassay (Bender MedSystems GmbH, Vienna, Austria) following the manufacturer's recommendations. Analysis was performed using a Cytomics FC500-MPL cytometer (Beckman Coulter) and the FlowCytomix Pro2.2.1 program from Bender MedSystems GmbH.

Quantification of secretory-immunoglobulin in small intestine

The small intestine was excised and carefully rinsed with ice-cold phosphate-buffered saline (PBS). The distal half of the small intestine was used to obtain the gut wash for secretory (S)-IgA and S-IgM quantification by enzyme-linked immunosorbent assay.¹²

Assessment of the inflammatory and oxidative status of the colon

The colon was excised and carefully rinsed with ice-cold PBS. The total weight and length of the colon were

determined to establish intestinal wall thickening and colon shortening, respectively. Thereafter, the colon was opened longitudinally and a transverse section of the damaged distal area was kept for histological analysis. The remaining colon was divided into four longitudinal strips that were immediately frozen in liquid N₂ and stored at -80°C to estimate some inflammatory mediators such as prostaglandin E₂ (PGE₂), leukotriene B₄ (LTB₄) and inducible nitric oxide synthase activity (iNOS). Thiobarbituric acid reactive substances (TBARS) and total glutathione (GSH) content were measured as antioxidant indicators.

A longitudinal colon stripe from each animal was homogenized in 0.1 mol/L sodium phosphate buffer pH 7.4 (200 g/L) containing 1 mmol/L ethylenediaminetetraacetic acid and 10 μ mol/L indomethacin. PGE₂ and LTB₄ concentrations were measured in diluted samples (1:10) using enzyme immunoassay kits (Cayman Chemicals, Tallinn, Estonia) according to the manufacturer's recommendations. Results were expressed as ng/g of wet tissue.

iNOS activity was measured using a commercial kit (Cayman Chemicals). For this purpose, a second longitudinal colon stripe from each animal was homogenized in the buffer sample provided by the kit, and the assay was performed following the manufacturer's instructions. Data were expressed as a conversion percentage of [³H] arginine to [³H] citrulline.

A third longitudinal colon stripe from each animal was homogenized in 1.15% KCl buffer containing 1% (v/v) butylhydroxytoluene at 100 g/L. TBARS concentration was determined as an index of lipid peroxidation, a major indicator of oxidative stress. Malondialdehyde (MDA) was used as a standard curve. Data were expressed as μ mol MDA/g of wet tissue.

A fourth longitudinal colon stripe from each animal was deproteinized by homogenization with 5% trichloroacetic acid in sodium phosphate buffer pH 6.0 (50 g/L), and followed by three repeated freeze-thaw cycles. GSH content was quantified using the 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB) recycling assay.¹⁸ DTNB was used as standard and data were expressed as μ mol/g of wet tissue.

Histological analysis

Colon segments were fixed in 10% buffered formaldehyde. Tissue samples were washed with tap water, dehydrated in increasing concentrations of ethanol (70% for one hour, 96% for one hour $\times$ 3) and embedded in paraffin. Tissue sections were cut (4 μ m) on a microtome (Leitz, Wetzlar, Germany) and stained with the hematoxylin-eosin technique. Histological assessment was carried out in a blind fashion considering cell infiltration and goblet cell loss together with structural changes. Microphotographs were taken with an Olympus BX41 microscope (Olympus, Hamburg, Germany).

Statistical analysis

The software package SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data were analyzed

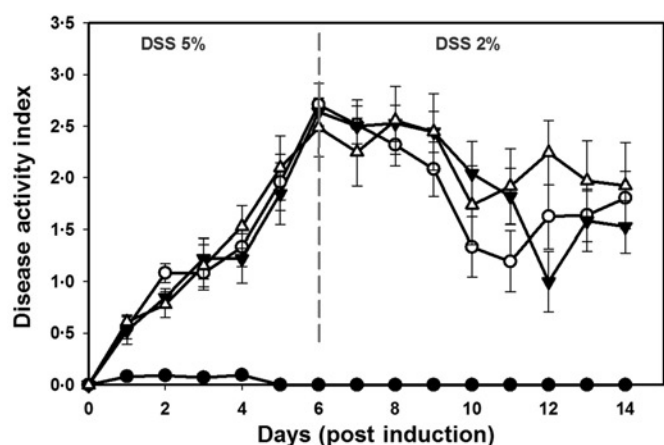


Figure 2 Clinical time course of dextran sulfate sodium (DSS)-induced colitis. Disease activity index was calculated by assessment of body weight, stool consistency and presence of blood in the stools for each animal, using a 0–4 severity scale. Values are means, with their standard errors represented by vertical bars ($n = 12$). —●—, Reference; —○—, DSS; —▼—, DSS-quercitrin; —△—, DSS-cocoa

using one-way analysis of variance (ANOVA) followed by the Scheffé's *post hoc* significance test after having confirmed normality and equal variance assumptions by the Kolmogorov–Smirnov's and Levene's statistical tests, respectively. Differences were considered statistically significant at P values of <0.05 .

Results

Effects of cocoa diet on clinical signs of colitis

Rats receiving DSS developed colitis, which was assessed by the DAI (Figure 2). DAI increased during the first six days, remained stable for the following three days and decreased from day 9. No significant differences were observed among the DSS-C, DSS-Q and DSS groups.

Effects of cocoa diet on colon architecture after receiving DSS

The analysis of the intestine after 14 days of DSS treatment revealed a higher weight and a shorter length of the colon in the DSS, DSS-Q and DSS-C groups in comparison with that of the REF group. In consequence, the colon weight/length ratio was significantly increased in the three groups ($P < 0.05$; Figure 3a) without significant differences among them. The cocoa diet and the quercitrin treatment did not prevent the changes induced by DSS in the colon architecture. However, the microscopic analysis of the colon sections from animals of the DSS-Q and DSS-C groups showed only a moderate lamina propria mononuclear cell infiltration and the prevention of goblet cell loss in comparison with the DSS group (Figures 3b and e).

Effects of cocoa diet on inflammatory and oxidant indicators from blood and colon

After 14 days of DSS treatment, the colonic inflammatory process was reflected by a marked leukocytosis in the

DSS, DSS-Q and DSS-C groups when compared with the REF group ($P < 0.05$; Figure 4a). This increase was due to both neutrophil and lymphocyte counts (Figures 4b and c), which were significantly higher in the DSS group than in the REF group ($P < 0.05$) and showed the same increased pattern in animals fed the cocoa diet. The leukocytosis in the DSS-Q group was mainly attributed to increased neutrophil counts since lymphocyte counts were similar to those found in the REF group.

Serum TNF- α concentration significantly increased in the DSS group compared with REF animals after 14 days of DSS drinking ($P < 0.05$; Table 2). Similar results were obtained in the DSS-Q group, which was treated with quercitrin. Interestingly, serum TNF- α increase was significantly prevented by the cocoa diet ($P < 0.05$).

The inflammatory response was also studied in colon homogenates by means of PGE₂ and LTB₄ concentrations and iNOS activity (Table 2). The PGE₂ concentration was higher in the DSS group ($P < 0.05$) than in the REF group, and neither the cocoa diet nor the quercitrin treatment prevented such increase. Similarly, the LTB₄ concentration was increased in the DSS group compared with REF animals and no effect was detected in the cocoa diet group. However, quercitrin treatment tended to decrease the LTB₄ concentration. On the other hand, colon iNOS activity was slightly increased in the DSS and DSS-Q groups compared with that of the REF group, but, interestingly, cocoa-fed animals inhibited iNOS activity in the colon that was even lower than in the REF animals ($P < 0.05$).

With regards to oxidative status in the colon, we determined TBARS as a measure of lipid peroxidation and GSH content as an endogenous antioxidant. Both the quercitrin treatment and the cocoa diet tended to reduce the TBARS produced in the DSS group (Table 2). GSH content was significantly decreased in the DSS group compared with the REF group ($P < 0.05$). The cocoa diet increased the colon GSH content ($P < 0.05$ versus DSS group), which was even higher than in the REF group. GSH content in the DSS-Q group was between that observed in the DSS and REF animals.

Effects of cocoa diet on MLNs and blood lymphocyte composition

In MLNs, the colon inflammation in the DSS group did not modify the percentage of Th, Tc, B, natural killer (NK), NKT, T_{reg} and T_{act} lymphocytes compared with the REF group (Figures 5a and c). However, both the quercitrin-treated and cocoa-fed groups displayed a lower Th cell percentage than the REF group ($P < 0.05$), and the DSS-C group showed a higher B-cell proportion ($P < 0.05$) than the REF group.

In the blood, DSS administration did not significantly alter the proportion of Th, Tc and B cells (Figure 5d) but did increase the percentages of NK and T_{reg} cells, which were higher in the three DSS groups than in the REF group ($P < 0.05$, Figures 5e and f). The DSS-Q group showed the highest T_{reg} proportion in Th cells and both the DSS-Q and DSS-C groups increased the proportion of T_{act} subset in Th cells ($P < 0.05$).

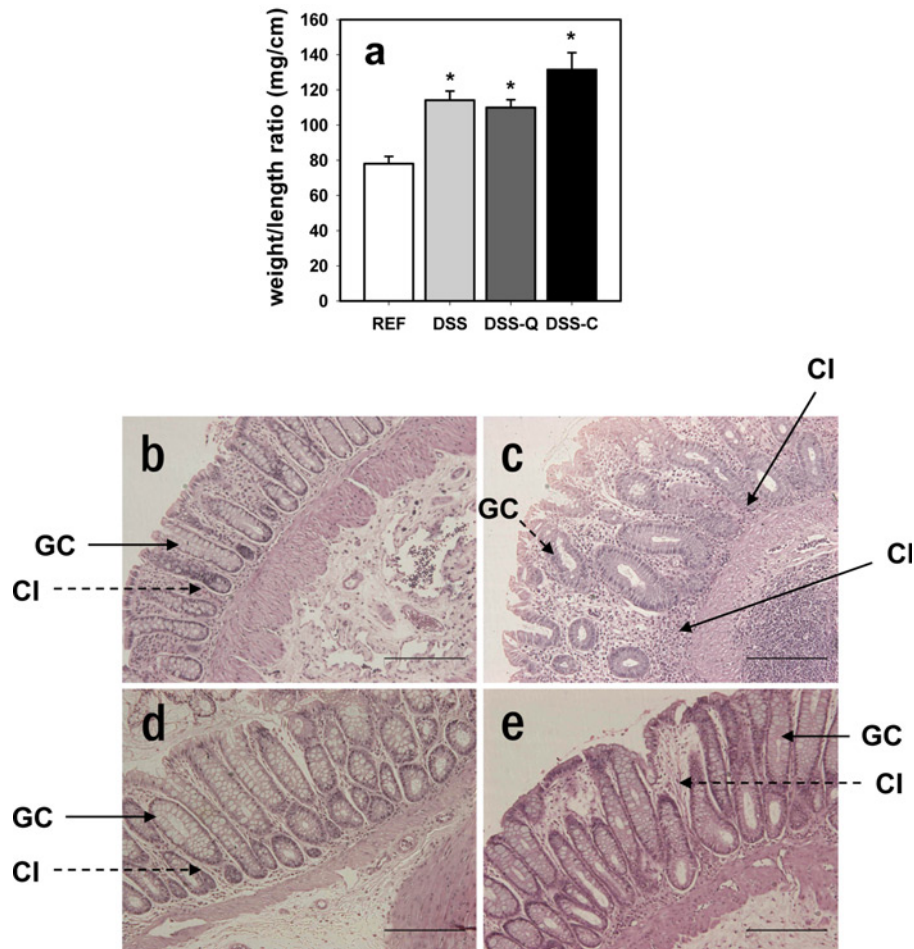


Figure 3 Colon architecture after dextran sulfate sodium (DSS) treatment. Colon weight/length ratio (a): values are means, with their standard errors represented by vertical bars ($n = 12$). *Mean values were significantly different from those of the reference (REF) group ($P < 0.05$; analysis of variance followed by Scheffé's *post hoc* significance test). Micrographs showing histological sections of colonic mucosa at the end of the study from a representative REF rat (b), a DSS control rat (c), a quercitrin-treated rat (d) and a cocoa-fed rat (e) (scale bar corresponds to $200 \mu\text{m}$). Continuous arrows indicate the presence of either goblet cells (GC) or cell infiltration (CI) and discontinuous arrows indicate their absence. DSS-Q, DSS-quercitrin; DSS-C, DSS-cocoa. (A color version of this figure is available in the online journal)

Effect of cocoa diet on small intestine S-IgA and S-IgM content

In the DSS group, the S-IgA content in the small intestine wash was not modified by the inflammatory process (Figure 6), although there was a reduction in the S-IgM content

compared with the REF animals ($P < 0.05$). This reduction was not observed in animals treated with quercitrin. The cocoa diet significantly reduced the amount of both S-IgA and S-IgM in the small intestine wash ($P < 0.05$) in comparison with the REF and the DSS groups.

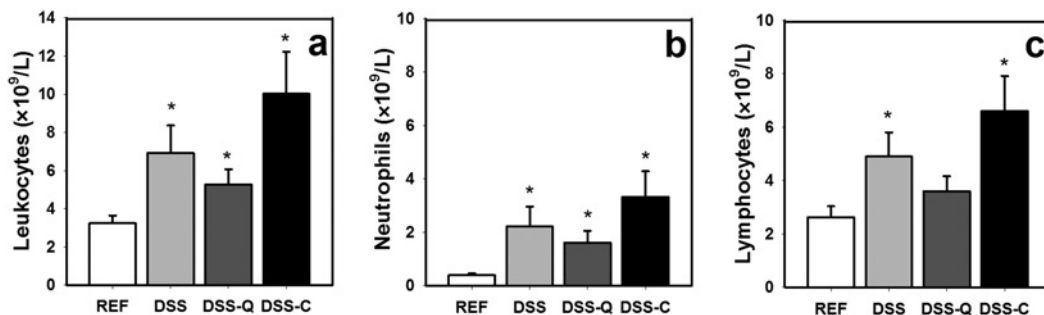


Figure 4 Blood leukocyte counts after dextran sulfate sodium (DSS) treatment. Number of total leukocytes (a), neutrophils (b) and lymphocytes (c). Values are means, with their standard errors represented by vertical bars ($n = 12$). *Mean values were significantly different from those of the reference (REF) group ($P < 0.05$; analysis of variance followed by Scheffé's *post hoc* significance test). DSS-Q, DSS-quercitrin; DSS-C, DSS-cocoa

Table 2 Inflammatory and oxidative status indicators in serum and colon specimens of studied groups

	REF	DSS	DSS-Q	DSS-C
Serum TNF- α (pg/mL)	13.4 $\pm$ 0.6	19.3 $\pm$ 1.8*	18.9 $\pm$ 1.8*	8.4 $\pm$ 1.0 [†]
Colon PGE ₂ (ng/g)	29.03 $\pm$ 3.95	46.14 $\pm$ 14.36*	58.75 $\pm$ 9.29*	49.31 $\pm$ 7.77*
Colon LTB ₄ (ng/g)	0.49 $\pm$ 0.07	0.68 $\pm$ 0.08*	0.59 $\pm$ 0.19	0.64 $\pm$ 0.13
Colon iNOS (%)	16.40 $\pm$ 1.84	18.90 $\pm$ 2.99	17.00 $\pm$ 2.03	11.5 $\pm$ 1.75 [†]
Colon TBARS (μ mol MDA/g)	2.12 $\pm$ 0.24	2.82 $\pm$ 0.44*	1.97 $\pm$ 0.27	2.26 $\pm$ 0.44
Colon GSH (μ mol/g)	0.96 $\pm$ 0.08	0.72 $\pm$ 0.06*	0.89 $\pm$ 0.12	1.05 $\pm$ 0.16 [†]

PGE₂, prostaglandin E₂; LTB₄, leukotriene B₄; iNOS, inducible nitric oxide synthase activity; TBARS, thiobarbituric acid reactive substance; GSH, total glutathione; REF, reference; DSS, dextran sulfate sodium; Q, quercitrin; C, cocoa; TNF- α , tumor necrosis factor- α ; MDA, malondialdehyde

Data are expressed as mean $\pm$ SD, $n = 12$

* $P < 0.05$ versus the REF group

[†] $P < 0.05$ versus the DSS group

Discussion

This paper assesses the impact of a dietary intervention with cocoa, as a source of antioxidant flavonoids, on the DSS model of rat colitis. In particular, we focused on the intestinal and systemic inflammatory response, including the oxidative status. A cocoa diet has been reported to show anti-inflammatory and immunomodulatory properties^{11–13} but there is a scarce number of studies describing the effects of cocoa on IBD. The effects observed were compared with those exerted by quercitrin, with recognized antioxidant and anti-inflammatory properties.⁶ The results demonstrate that in our experimental conditions, cocoa intake and quercitrin treatment do not

improve clinical colitis, although they certainly contributed to reducing colonic oxidative activity and tissue damage. Moreover, cocoa showed anti-inflammatory potential by down-regulating serum TNF- α concentration and colon iNOS activity and by decreasing colon cell infiltration.

The pathogenesis of IBD involves the recruitment of leukocytes into the intestinal mucosa.¹⁹ Among them, macrophages secrete large amounts of inflammatory mediators and nitrogen and reactive oxygen species that induce oxidative stress.¹⁹ In this line, we found that DSS-induced colitis produced consumption of glutathione and an increased lipid peroxidation in the colon. The cocoa diet avoided the glutathione consumption and

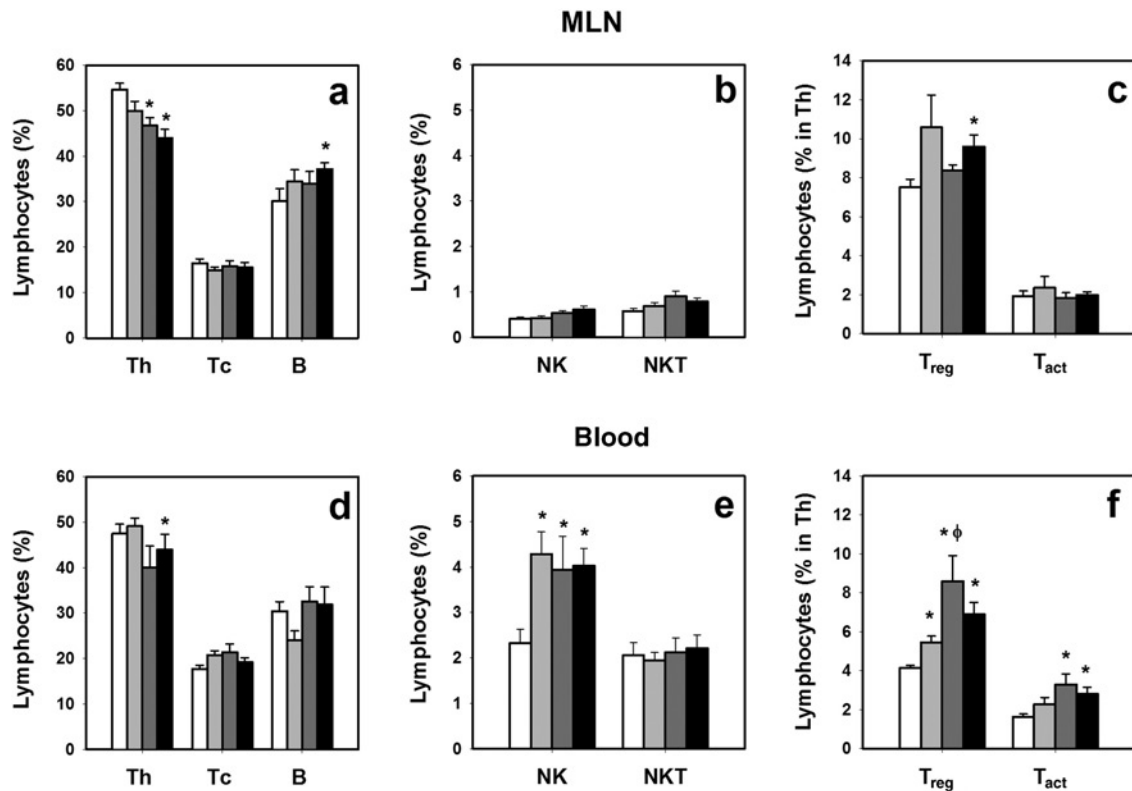


Figure 5 Mesenteric lymph node (MLN) (a–c) and blood (d–f) lymphocyte composition after dextran sulfate sodium (DSS) treatment. Percentages of Th, Tc and B-cells (a, d), natural killer (NK) and NKT subsets (b, e), T_{reg} and T_{act} cells in Th subset (c, f). Values are means, with their standard errors represented by vertical bars ($n = 12$). *Mean values were significantly different from those of the reference (REF) (□) group ($P < 0.05$; analysis of variance [ANOVA] followed by Scheffé's *post hoc* significance test). †Mean values were significantly different from those of the DSS (■) group ($P < 0.05$; ANOVA followed by Scheffé's *post hoc* significance test). ■ DSS-quercitrin group; ■ DSS-cocoa group

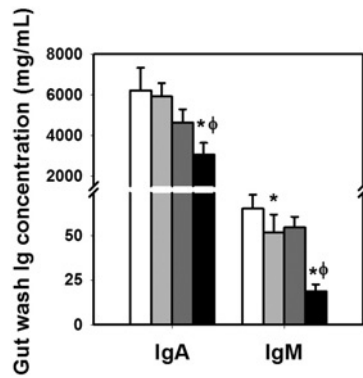


Figure 6 Secretory (S)-IgA and S-IgM concentrations in the small gut wash. Values are means, with their standard errors represented by vertical bars ($n = 12$). *Mean values were significantly different from those of the reference (REF) (□) group ($P < 0.05$; analysis of variance [ANOVA] followed by Scheffé's *post hoc* significance test). *Mean values were significantly different from those of the dextran sulfate sodium (DSS, ■) group ($P < 0.05$; ANOVA followed by Scheffé's *post hoc* significance test). ▒ DSS-quercitrin group; ■ DSS-cocoa group

partially prevented the lipid peroxidation. Similar results were obtained by quercitrin treatment, although neither the cocoa diet nor the quercitrin treatment reduced the clinical development of colitis. In agreement with our results, the antioxidant luteolin incorporated into the diet does not ameliorate and even worsens the DSS-induced colitis in NF- κ B^{EGFP} transgenic mice.²⁰ Moreover, consumption of a lemon verbena infusion rich in polyphenolic compounds did not improve the histopathological changes induced by 4% DSS in rats, despite stimulating the activity of the antioxidant enzyme superoxide dismutase and reducing lipid peroxidation in the colon.²¹ However, it has been recently reported that a polyphenol-enriched cocoa extract was able to decrease acute DSS colitis in mice,²² thus evidencing the promising anti-inflammatory effects of cocoa flavonoids. These results may also suggest that we did not observe a clear colitis reduction because of the lower polyphenol content in the cocoa that we used.

On the other hand, as we have confirmed here, colitis is associated with increases in the inflammatory mediators LTB₄ and PGE₂.²³ However, neither cocoa nor quercitrin treatment could avoid such increases. Another indicator of the inflammatory process in the colon and macrophage infiltrates is the iNOS activity.⁶ Although we found no higher iNOS activity in animals receiving DSS, interestingly, the cocoa-fed group showed lower iNOS activity than the DSS group. Similarly, the cocoa diet prevented the increase in serum TNF- α concentration, a key molecule in IBD pathogenesis.²⁴ Both protective effects on iNOS and TNF- α are in line with previous *in vitro* and *in vivo* studies,^{11,25} but they could not contribute to attenuate the intestinal inflammatory process.

In order to assess the effects of cocoa on the inflammatory process beyond the colon, cell composition was established in the blood and MLNs. There is limited information about the impact of DSS on the cell populations of these compartments in rat. In our study, the inflammatory process did not vary the proportion of Th, Tc, B, NK, NKT, T_{reg} and T_{act} lymphocytes in the MLNs. However, the cocoa diet

increased the proportion of B lymphocytes, and both the cocoa diet and the quercitrin treatment reduced the proportion of Th cells in the MLNs. The results from the cocoa intake by the colitic animals are in line with the effects observed in the MLNs of cocoa-fed healthy animals.¹³ In any case, the decrease of the proportion of Th cells did not involve clinical recovery, which could be attributed to the fact that Th or B-cells may not be essential in DSS colitis, as shown in immunodeficient mice.²⁶ On the other hand, we found no significant changes in the MLN percentage of T_{reg} cells, contrary to recent studies showing a decrease of T_{reg} subset in the MLNs of mice that received 5% DSS in their drink for seven days.²⁷

In the blood compartment, we found that the intestinal inflammatory process produced neutrophilia and lymphocytosis but the proportions of the main lymphocyte subsets were not modified. However, the NK cell percentage increased in the blood of the DSS animals. This finding is consistent with the fact that depletion of NK cells attenuates the severity of acute colitis in DSS-treated mice²⁸ and suggests an important role of these cells in colitis. Neither the cocoa diet nor the quercitrin treatment prevented the increase of the blood NK cell proportion, which could be one of the reasons why cocoa-fed animals did not display colitis amelioration in spite of showing antioxidant activity in the colon and reduced serum TNF- α . Regarding the T_{reg} subpopulation, the inflammatory process unexpectedly increased the percentage of T_{reg} cells in Th lymphocytes in the blood. The T_{reg} increase might be seen as an attempt to re-establish intestinal homeostasis under inflammatory conditions.

Although the exact mechanism through which DSS initiates colitis remains unknown, different studies suggest that DSS administration may compromise the gut epithelial barrier function, resulting in an increased permeability.¹⁴ The mucosal IgA system contributes to the barrier functionality, generating immune protection in a non-inflammatory manner. The cocoa diet significantly decreased the S-IgA and S-IgM in the small gut as previously reported in healthy animals.¹³ Although in our study the colonic S-IgA concentration was not assessed, the down-regulatory effect on S-IgA of the small intestine may have also occurred in the colon of cocoa-fed animals. In fact, previous studies have reported decreased S-IgA concentration in the feces of healthy animals fed with a diet containing 5% of cocoa.²⁹ Therefore, it could be suggested that the cocoa diet, although exerting antioxidant and even anti-inflammatory protection, may increase colon permeability by reducing S-IgA content, which may contribute to the inflammatory process. In other words, those beneficial effects of cocoa flavonoids may have been counteracted by a decreased S-IgA protection in the colon.

In conclusion, a diet containing cocoa may help to manage the oxidative stress consequent to colitis, although this action is not enough to reverse the inflammatory process. Cocoa intake shows anti-inflammatory potential and seems to decrease colon cell infiltration. Therefore, the cocoa diet may be considered as a promising complementary therapy for IBD.

Author contributions: TP-B carried out the study, performed the statistical analysis and drafted the manuscript. CR-S, SR-R and CC collaborated during the *in vivo* part and the discussion of data. MC, AF and FJP-C conceived and designed the study and discussed the results.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Spanish Ministry of Science and Innovation (AGL2005-02823). The authors thank Dr Ruth Ferrer for her technical assistance in the performance of the radiolabelled assay and María Pérez-Cano for the preparation of the histological sections. They also thank Dr Mónica Comalada at the University of Granada for her expert advice in establishing the DSS colitis.

REFERENCES

- Strober W, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. *J Clin Invest* 2007;**117**:514–21
- Danese S. Immune and nonimmune components orchestrate the pathogenesis of inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol* 2011;**300**:G716–22
- Hudert CA, Weylandt KH, Lu Y, Wang J, Hong S, Dignass A, Serhan CN, Kang JX. Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. *Proc Natl Acad Sci USA* 2006;**103**:11276–81
- Ancha HR, Kurella RR, McKimney CC, Lightfoot S, Harty RF. Luminal antioxidants enhance the effects of mesalamine in the treatment of chemically induced colitis in rats. *Exp Biol Med* 2008;**233**:1301–8
- Ye Z, Liu Z, Henderson A, Lee K, Hostetter J, Wannemuehler M, Hendrich S. Increased CYP4B1 mRNA is associated with the inhibition of dextran sulfate sodium-induced colitis by caffeic acid in mice. *Exp Biol Med* 2009;**234**:605–16
- Camuesco D, Comalada M, Rodríguez-Cabezas ME, Nieto A, Lorente MD, Concha A, Zarzuelo A, Gálvez J. The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression. *Br J Pharmacol* 2004;**143**:908–18
- Gu L, House SE, Wu X, Ou B, Prior RL. Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. *J Agric Food Chem* 2006;**54**:4057–61
- Saura-Calixto F. Dietary fiber as a carrier of dietary antioxidants: an essential physiological function. *J Agric Food Chem* 2011;**59**:43–9
- Urpi-Sardà M, Monagas M, Khan N, Lamuela-Raventos RM, Santos-Buelga C, Sacanella E, Castell M, Permanyer J, Andrés-Lacueva C. Epicatechin, procyanidins, and phenolic microbial metabolites after cocoa intake in humans and rats. *Anal Bioanal Chem* 2009;**394**:1545–56
- Natsume M, Osakabe N, Yasuda A, Baba S, Tokunaga T, Kondo K, Osawa T, Terao J. *In vitro* antioxidative activity of (–)-epicatechin glucuronide metabolites present in human and rat plasma. *Free Radic Res* 2004;**38**:1341–8
- Ramiro E, Franch A, Castellote C, Pérez-Cano F, Permanyer J, Izquierdo-Pulido M, Castell M. Flavonoids from *Theobroma cacao* down-regulate inflammatory mediators. *J Agric Food Chem* 2005;**53**:8506–11
- Ramos-Romero S, Perez-Cano FJ, Castellote C, Castell M, Franch A. Effect of cocoa-enriched diets on lymphocytes involved in adjuvant arthritis in rats. *Br J Nutr* 2012;**107**:378–87
- Ramiro-Puig E, Pérez-Cano FJ, Ramos-Romero S, Pérez-Berezo T, Castellote C, Permanyer J, Franch A, Izquierdo-Pulido M, Castell M. Intestinal immune system of young rats influenced by cocoa-enriched diet. *J Nutr Biochem* 2008;**19**:555–65
- Solomon L, Mansor S, Mallon P, Donnelly E, Hoper M, Loughrey M, Kirk S, Gardiner K. The dextran sulphate sodium (DSS) model of colitis: an overview. *Comp Clin Pathol* 2010;**19**:235–9
- Ramiro-Puig E, Urpi-Sardà M, Pérez-Cano FJ, Franch A, Castellote C, Andrés-Lacueva C, Izquierdo-Pulido M, Castell M. Cocoa-enriched diet enhances antioxidant enzyme activity and modulates lymphocyte composition in thymus from young rats. *J Agric Food Chem* 2007;**55**:6431–8
- Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. *J Nutr* 1997;**127**:838S–41S
- Cooper HS, Murthy SNS, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest* 1993;**69**:238–50
- Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* 1985;**113**:548–55
- Kruidenier L, Kuiper I, Lamers CBHW, Verspaget HW. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. *J Pathol* 2003;**201**:28–36
- Karrasch T, Kim JS, Jang BI, Verspaget HW. The flavonoid luteolin worsens chemical-induced colitis in NF- κ BEGFP transgenic mice through blockade of NF- κ B-dependent protective molecules. *PLoS One* 2007;**2**:e596
- Lenoir L, Rossary A, Joubert-Zakeyeh J, Vergnaud-Gauduchon J, Farges MC, Fraisse D, Texier O, Lamaison JL, Vasson MP, Felgines C. Lemon verbena infusion consumption attenuates oxidative stress in dextran sulfate sodium-induced colitis in the rat. *Dig Dis Sci* 2011;**56**:3534–45
- Andújar I, Recio MC, Giner RM, Cienfuegos-Jovellanos E, Laghi S, Muguerza B, Rios JL. Inhibition of ulcerative colitis in mice after oral administration of a polyphenol-enriched cocoa extract is mediated by the inhibition of STAT1 and STAT3 phosphorylation in colon cells. *J Agric Food Chem* 2011;**59**:6474–83
- Sharma JN, Mohammed LA. The role of leukotrienes in the pathophysiology of inflammatory disorders: is there a case for revisiting leukotrienes as therapeutic targets? *Inflammopharmacology* 2006;**14**:10–6
- Ngo B, Farre CP, Barr M, Wolov K, Bailey R, Mullin JM, Thornton JJ. Tumor necrosis factor blockade for treatment of inflammatory bowel disease: efficacy and safety. *Curr Mol Pharmacol* 2010;**3**:145–52
- Ramos-Romero S, Pérez-Cano FJ, Pérez-Berezo T, Castellote C, Franch A, Castell M. Effect of a cocoa flavonoid-enriched diet on experimental autoimmune arthritis. *Br J Nutr* 2012;**107**:523–32
- Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* 1994;**107**:1643–52
- Yao J, Wang JY, Lai MG, Li YX, Zhu HM, Shi RY, Mo J, Xun AY, Jia CH, Feng JL, Wang LS, Zeng WS, Liu L. Treatment of mice with dextran sulfate sodium-induced colitis with human interleukin 10 secreted by transformed *Bifidobacterium longum*. *Mol Pharm* 2011;**8**:488–97
- Yoshihara K, Yajima T, Kubo C, Yoshikai Y. Role of interleukin 15 in colitis induced by dextran sulphate sodium in mice. *Gut* 2006;**55**:334–41
- Pérez-Berezo T, Franch A, Ramos-Romero S, Castellote C, Pérez-Cano FJ, Castell M. Cocoa-enriched diets modulate intestinal and systemic humoral immune response in young adult rats. *Mol Nutr Food Res* 2011;**55**:S56–S66

(Received March 6, 2012, Accepted July 18, 2012)