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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

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ABSTRACT: We studied a polyphenol-enriched cocoa extract (PCE) with epicatechin, procyanidin B2, catechin, and procyanidin B1 as the major phenolics for its anti-inflammatory properties against dextran sulfate sodium (DSS)-induced ulcerative colitis (UC) in mice. PCE reduced colon damage, with significant reductions in both the extent and the severity of the inflammation as well as in crypt damage and leukocyte infiltration in the mucosa. Analysis ex vivo showed clear decreases in the production of nitric oxide, cyclooxygenase-2, pSTAT-3, and pSTAT1α, with NF-κB p65 production being slightly reduced. Moreover, NF-κB activation was reduced in RAW 264.7 cells in vitro. In conclusion, the inhibitory effect of PCE on acute UC induced by DSS in mice was attenuated by oral administration of PCE obtained from cocoa. This effect is principally due to the inhibition of transcription factors STAT1 and STAT3 in intestinal cells, with NF-κB inhibition also being implicated.

KEYWORDS: *Theobroma cacao*, cocoa, polyphenols, inflammatory bowel disease, ulcerative colitis

INTRODUCTION

The term inflammatory bowel disease (IBD) refers to a chronic, relapsing, and remitting inflammatory disorder with a multifactorial etiology and which is characterized by the presence in the gut of extensive areas of ulceration, pronounced infiltration of neutrophils, and epithelial cell necrosis. The main manifestations of the disease are ulcerative colitis (UC) and Crohn's disease (CD),[1] although there are other less common types such as Behçet's syndrome and lymphocytic, infectious, or ischemic colitis. Although the exact pathogenesis of IBD is poorly understood, infections, environmental factors, complex genetic disorders, and a deregulated immune response have all been proposed as causes.[1] As the name suggests, UC is limited to the colon, while CD can involve any part of the gastrointestinal tract from the mouth to the anus, but most commonly affects the small intestine and/or the colon.

Depending on the degree of severity, different treatments are used against IBD, although none of them are directly focused on healing the disease, but rather on attenuating its symptoms. Corticosteroids such as prednisone and 5-aminosalicylic acid are standard first line therapies for the induction of remission in mild to moderate IBD. 5-Aminosalicylic acid not only inhibits cyclooxygenase and lipoxygenase, but may also function as an agonist of peroxisome proliferator-activated receptor (PPAR)-γ, which inhibits inflammatory pathways dependent on nuclear factor (NF)-κB.[2] Once remission is achieved, a maintenance treatment based on immunomodulators such as infliximab or azathioprine is established. However, current IBD therapy displays limited beneficial action, and ulcerations of the colon eventually evolve into fistulas, perforation, and bleeding, which can only be treated through resection of the colon.

Natural products and dietary components are becoming an attractive approach for treating various inflammatory disorders among patients who are either unresponsive or who develop significant side effects to the treatment drugs. Plant polyphenols constitute one of the largest and most ubiquitous groups of secondary metabolites forming an integral part of the human diet. In humans, the average daily intake of polyphenols is higher than 1 g/day, with polyphenol concentrations in the gastrointestinal tract sometimes reaching levels of up to several hundred micromolars.[3] Shapiro et al.,[4] on the basis of a review of several animal models of acute and chronic colitis treated with different polyphenols, suggested that dietary polyphenols could be used as a supportive therapy for IBD patients. Moreover, several human trials have shown correlations between polyphenol intake (particularly flavonoids) and reduced incidence of cancer and cardiovascular and neurodegenerative diseases, as well as general benefits to gut health through inhibition of the growth of pathogenic gut bacteria and modulation of inflammatory conditions in the bowel.[5]

NF-κB is considered to be a good target for treating diseases with an inflammatory component. Many polyphenols attenuate in vivo activation of this transcription factor by inhibiting IKK phosphorylation and/or preventing IκB phosphorylation and degradation. Examples include kaempferol and quercetin, found in apples, onions, and leafy green vegetables; epigallochatechin

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gallate from green tea; curcumin, the major anti-inflammatory phenolic in turmeric; and resveratrol, found in grapes and wine.

Cocoa has one of the highest flavanol contents of all the different polyphenol-rich foods.[6] The flavanols in cocoa, derived from the processed seeds of *Theobroma cacao* L. (Sterculiaceae), are a class of flavonoids that include (−)-epicatechin and (+)-catechin, as well as oligomers of these molecules, which are called procyanidins. Cocoa polyphenols have been reported to exhibit several health benefits; in particular, their beneficial cardiovascular effects have been studied extensively.[7] Indeed, the consumption of cocoa has been found to improve endothelial function and to reduce pro-inflammatory mediators.[8] Although polyphenol bioavailability is relatively poor in general,[3] flavanols with low molecular weight are among the most bioavailable flavonoid compounds. In fact, cocoa flavonoids have been shown to be bioavailable in humans.[9] Recent research has thus focused on obtaining new products rich in flavan-3-ol and oligomeric procyanidins of low molecular weight since different studies have reported that the health properties attributed to cocoa seem to be related to its high content in monomeric and dimeric compounds.[7] In a previous study, we reported on a new process for developing cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans.[10] This flavonoid-enriched cocoa powder contained four times more procyanidins and eight times more epicatechin and procyanidin B2 than conventional cocoa powder. It has since been characterized and its bioavailability and antihypertensive effects have been described.[11,12] On the other hand, the use of high polyphenol content of enriched cocoa extract allows consumers to reach the polyphenol dosage necessary for obtaining a health benefit with less product. Another great advantage of extracts is their ease of use in the food industry due to their physicochemical properties, such as their high solubility. Furthermore, as the quantity added to the final application is quite small, the organoleptic profile of the final food is not significantly altered by the ingredient. Because of this, a polysphenol-enriched cocoa extrac (PCE) could be used for multiple applications, not just those traditionally associated with the consumption of cocoa polyphenols. In this study, we examine the effect of oral administration of a PCE on an animal model of ulcerative colitis, and its possible mechanisms of action in order to establish its potential properties as a nutraceutical or pharmacological agent.

MATERIALS AND METHODS

Polyphenol-Enriched Cocoa Extract (PCE). An industrial process is used to preserve cocoa's natural content in polyphenols. PCE, a hygroscopic red-violet powder soluble in water at room temperature at 10 g/L, was obtained from CocoanOx, a polyphenol-enriched cocoa powder produced from unfermented, blanch-treated, and nonroasted cocoa beans using the procedure described by Cienfuegos-Jovellanos et al.[13] After procuring the PCE, we determined its theobromine content by conducting duplicate tests with high performance liquid chromatography (HPLC).[14] The total polyphenol content was determined with Folin−Ciocalteu's spectrophotometric method and was expressed using catechin equivalents on a wet weight basis.[15]

Flavan-3-ols, (+)-catechin, (−)-epicatechin, and procyanidins B2 and B1 were identified and quantified by means of reverse-phase (RP)-HPLC in an Agilent 1100 Series chromatograph equipped with a diode array detector and ChemStation for data collection and manipulation, as described elsewhere.[11] The results are reported on a wet weight basis. Standards of (+)-catechin, (−)-epicatechin (Sigma-Aldrich, St. Louis, MO, USA), and procyanidins B1 and B2 (Extrasynthese, Genay, France) were used for quantitative determinations. HPLC-grade organic solvents were purchased from Scharlab (Barcelona, Spain) and Merck (Darmstadt, Germany). Standard solutions were prepared daily and stored in the refrigerator at 4 °C. Prior to injection, the solutions were filtered through a 0.45 μm PTFE filter (Teknokroma, Barcelona, Spain). Theobromine standard was purchased from Avocado-Panreac (Barcelona, Spain).

Procyanidin fractions (monomer fraction through polymers) were analyzed with the aid of normal-phase HPLC-MS as previously described.[16]

Table 1. Scoring System to Calculate the Disease Activity Index (DAI)[a]

scoring of disease activity index			
score	weight loss	stool consistency	visible blood in feces
0	none	normal	none
1	1−5%		
2	6−10%	loose	slight bleeding
3	11−20%		
4	>20%	diarrhea	gross bleeding

[a] The DAI value is calculated as the sum of the scores for weight loss, stool consistency, and blood in feces.

Animals. Female Balb/C mice weighing 18−20 g (Harlan Interfauna Ibérica, Barcelona, Spain) were used for in vivo experiments. All animals were fed a standard diet ad libitum and housed under a 12-h light/dark cycle at 22 °C and 60% humidity. Housing conditions and all in vivo experiments were approved by the Institutional Ethics Committee of the University of Valencia, Spain.

Induction of Acute Ulcerative Colitis and Treatment with PCE. Three groups of 7−10 animals each were used. Acute colitis was induced in two of the groups through oral administration of 5% dextran sulfate sodium (DSS, w/v) in fresh tap water ad libitum for seven days. The first group received no further treatment. The second group was treated with 500 mg/kg of PCE administered orally with the aid of a gastric probe at the beginning of the experiment and then again four days later. The third group, which received neither DSS nor PCE, was given only fresh tap water. Water consumption was controlled for all groups, and no major differences were detected. The disease activity index was determined according to the parameters outlined in Table 1.[17] At the end of the seven day experiment period, the mice were sacrificed by means of cervical dislocation; their colonic tissues were dissected for further analysis. This same protocol was carried out three times in independent experiments.

Culture of Colon Organ Cells. After each in vivo protocol, the distal colon of three mice was removed, opened longitudinally, and washed in phosphate-buffered saline (PBS), as described elsewhere.[18] The colon was then further cut into 1 cm segments and cultured for 24 h in a 24-well plate with complete Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U per mL), and streptomycin (100 U per mL).

Mouse Peritoneal Macrophage Isolation. Untreated Balb/C mice were given a 2 mL intraperitoneal injection of 3% thioglycollate in water. Four days later, mouse peritoneal macrophages were collected through peritoneal lavage with PBS, pelleted, and washed in PBS. Cells were plated at a density of 1×10^6 cells per mL with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U per mL), and streptomycin (100 U per mL). Cells were given 2 h to adhere, after which the medium was changed, and adherent cells were pretreated for 1 h with PCE (5 and 25 μg/mL) and stimulated with LPS (1 μg/mL) for 24 h without removing the PCE

Table 2. Histological Scoring System for DSS-Induced Colitis[a]

scoring of severity of histological damage		
feature	score	description
severity of inflammation	0	none
	1	mild
	2	moderate
	3	severe
extent of inflammation	0	none
	1	mucosa
	2	mucosa and submucosa
	3	transmural
crypt damage	0	none
	1	1/3 damaged
	2	2/3 damaged
	3	crypts lost, surface epithelium present
	4	crypts and surface epithelium lost

[a] Scores were calculated by adding the score for the three parameters, giving a maximum score of 10.

from the culture medium. The supernatant was used for cytokine and nitric oxide assays.

Measurement of Cytokines. The concentrations of various cytokines in the culture supernatants of the mouse colons and peritoneal macrophages were measured using an enzyme-linked immunosorbent assay kit (eBioscience, San Diego, CA, USA) in accordance with the manufacturer's instructions.

Nitric Oxide Determination with Griess Assay. Nitric oxide levels were assessed by means of nitrite quantification as described elsewhere.[19] Briefly, 100 μL of culture medium was incubated for 15 min with Griess reagent (Sigma-Aldrich). Absorbance was read at 540 nm.

Histology. Small (approximately 1 cm) sections of excised distal colonic tissue were fixed in 10% paraformaldehyde in PBS, pH 7.4, and embedded in paraffin. Sections (4 μm) were cut and stained with hematoxylin. Histological assessment of colonic mucosa was carried out in a blind fashion by an experienced pathologist as described previously by Tamaki et al.;[20] the results are given in Table 2.

Preparation of Cytosolic and Nuclear Fractions from Mouse Colons. Colon samples from the mid to distal colon were rinsed with cold PBS, blotted dry, and immediately frozen with liquid nitrogen. The samples were stored at −80 °C until use. Protein extraction from the intestine was performed as described previously by Sugimoto et al.[21] Briefly, tissues were thawed and ground to powder in a mortar and homogenized in 1.5 mL of ice-cold buffer A [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A]. Ipegal CA-630 was added to a final concentration of 0.5%. The homogenates were chilled on ice with gentle shaking for 45 min. The membrane fraction was precipitated by means of centrifugation at 106*g* for 10 min at 4 °C. The supernatant containing the cytosolic fraction was stored at −80 °C until use. The pellet was resuspended with vortex mixing in 500 μL of buffer B (20 mM HEPES pH 7.8, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A) and chilled for 30 min on ice with gentle shaking. After centrifugation at 20,800*g* for 15 min at 4 °C, the supernatant containing the nuclear fraction was removed and stored at −80 °C until use. Cell lysates (30 μg of protein) were boiled in sodium dodecyl sulfate sample buffer for 5 min before undergoing electrophoresis (see Western blot assay).

Myeloperoxidase (MPO) Activity Assay. Colon samples from the mid to distal colon were rinsed with cold PBS, blotted dry, and immediately frozen with liquid nitrogen. The samples were stored at −80 °C until use. For MPO determination, samples were ground to powder in a mortar. MPO was determined as described by Suzuki et al.[22] Briefly, approximately 40 mg of tissue from each sample was weighed and homogenized in 80 mM KH_2PO_4/Na_2HPO_4 buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). After centrifugation, 100 μL of PBS, 85 μL of 22 mM KH_2PO_4/Na_2HPO_4 buffer, and 15 μL of hydrogen peroxide 0.017% were added to 30 μL of the supernatant. The enzymatic reaction was then started by adding 20 μL of 3,3′,4,4′-tetramethylbenzidine hydrochloride. After 3 min at 37 °C, the reaction was stopped by the addition of 30 μL of 1.46 M sodium acetate buffer, pH 3, on ice. Absorbance was read at 630 nm. MPO activity was expressed as the amount of enzyme required to convert 1 μmol of hydrogen peroxide to water in 1 min, expressed per gram of wet weight of tissue.

Western Blot Analysis for Cyclooxygenase-2, NF-κB p65, and Phosphorylated Signal Transducer and Activator of Transcription (pSTAT)-3 and -1α. Western blot analyses were performed in both colon cytosolic and nuclear fractions. After extraction, the presence of proteins in the supernatants was determined with the aid of the Bradford method with bovine serum albumin as the standard. Equal amounts of protein (30 μg) were then loaded onto 10% sodium dodecyl sulfate−polyacrylamide electrophoresis gel and transferred onto polyvinylidene difluoride membranes at 125 mA for 90 min. The membranes were then blocked in PBS-Tween 20 containing 3% w/v defatted milk and, when needed, antiphosphatases (25 mM sodium fluoride and 2 mM sodium orthovanadate). For cyclooxygenase-2, the membranes were incubated with anticyclooxygenase-2 polyclonal antibody (1/1000 dilution) obtained from Cayman (Ann Arbor, MI, USA). For p65, the membranes were incubated with anti-p65 polyclonal antibody (1/500 dilution) (SC-7151); for pSTAT3, the membranes were incubated with anti-pSTAT3 polyclonal antibody (SC-8059) (1/500 dilution), while for pSTAT1α, anti-pSTAT1α polyclonal antibody (SC-7988) (1/500 dilution) was used. These three antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Finally, for β-actin, the membranes were incubated with anti-β-actin polyclonal antibody (1/12000 dilution) obtained from Sigma-Aldrich. The blots were washed and incubated with peroxidase-conjugate anti-rabbit, antimouse, or antigoat immunoglobulin G (1/20000 dilution) (Cayman). The immunoreactive bands were visualized with the aid of an enhanced chemiluminiscence system (Millipore Corporation, Billerica, MA, USA).

Cell Culture. The murine macrophage cell line RAW 264.7 (ECACC, Salisbury, UK) was maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin sulfate (100 μg/mL) in a humidified 5% CO_2 atmosphere.

Transient Transfection and NF-κB-Dependent Reporter Gene Expression Assay. To examine the effect of PCE on the transcriptional activity of NF-κB, RAW 264.7 cells (5×10^5 cells per well) were placed in 24-well plates and transiently transfected with pNF-κB-Luc expression plasmid (0.8 μg) and the control plasmid TK-Renilla (0.2 μg), both kindly donated by Dr. Lidija Klampfer (Albert Einstein Cancer Center, New York, USA). Transfections were performed with Lipofectamine 2000 following the manufacturer's instructions (Invitrogen, Paisley, UK). Twenty-four hours after transfection, the cells were pretreated with PCE for 1 h and then stimulated with LPS for 18 h without removing the PCE from the culture medium. The luciferase assay was performed with the aid of Dual-Luciferase Assay System following the manufacturer's instructions (Promega, Madison, WI, USA).

Luciferase activity was normalized to TK-Renilla activity to control for transfection efficiency.

Software. Images for all Western blot and Electrophoretic Mobility Shift Assay (EMSA) experiments were acquired with the LAS-3000 mini image analysis system (Fujifilm, Tokyo, Japan). Digital images were processed, and band density measurements were made with the aid of a Multi Gauge V3.0 software package (Fujifilm).

Statistical Analysis. Statistical analysis was performed with a one-way analysis of variance (ANOVA) and Dunnett's *t*-test or with a nonparametric post hoc test (Kruskal–Wallis test). The results are presented as the mean ± SEM. GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for all calculations.

Table 3. Theobromine, Polyphenol, and Procyanidin Profile of the Soluble Cocoa Fiber Product (mg/g Dry Matter)[a]

components (g/kg dry matter)[a]	
theobromine	96.7 ± 0.2
total polyphenol content[b]	546.7 ± 4.3
total flavan-3-ol content[c]	293.3 ± 80.5
procyanidin B1	9.7 ± 0.2
(+)-catechin	11.6 ± 1.1
procyanidin B2	84.3 ± 1.2
(−)-epicatechin	143.1 ± 1.2

[a] The values are expressed as the mean ± SD (n = 3). [b] Measured with Folin–Ciocalteu's method. [c] Measured with HPLC-diode-array detection (DAD).

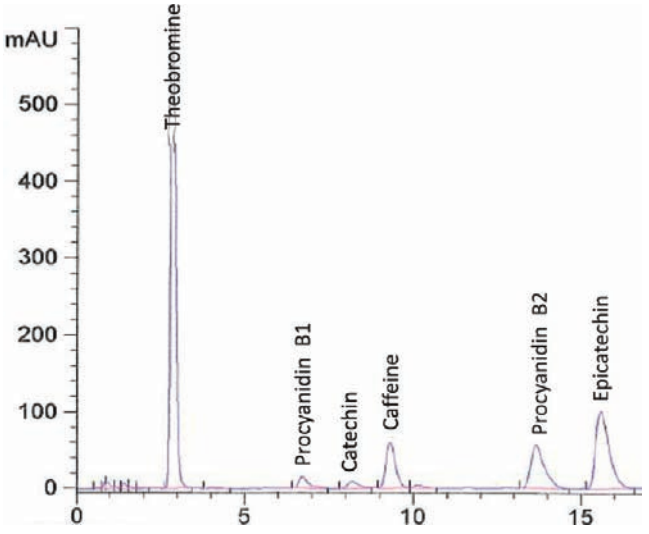

Figure 1. RP-HPLC-DAD chromatogram of flavan-3-ols of PCE.

RESULTS

Theobromine, total polyphenol, and flavan-3-ol contents of the sample used in this study are presented in Table 3, while Figure 1 shows the RT-HPLC-DAD profile of the different procyanidins present. The major phenolics identified in PCE were epicatechin, procyanidin B2, catechin, and procyanidin B1, together with theobromine. The procyanidins (ranging from monomers to polymers) quantified in PCE with the aid of normal-phase HPLC-MS are given in Table 4.

PCE clearly reduced the damage caused by DSS-induced acute ulcerative colitis in mice. Eight days after treatment, there was a significant reduction of weight loss, and colon shortening was also prevented as compared to that in the group which received only DSS (Figure 2). The effect of PCE on the disease activity index (DAI) score was evaluated for DSS groups and PCE-treated groups after the last day of treatment and scored as shown in Table 1. The PCE-treated mice showed a reduction in weight loss, normal stool consistency, and slight or no visible blood in feces, resulting in a lower total DAI score as compared to that of the DSS group (Figure 3). The histological damage was evaluated in three representative colonic hematoxylin samples of mice that received either fresh tap water (Figure 4A), fresh tap water with 5% DSS (Figure 4B), or fresh tap water with 5% DSS and PCE treatment (500 mg/kg) (Figure 4C). Significant reductions in both the severity and the extent of inflammation were observed, as well as a decrease in crypt damage. The severity of damage was scored as described in Table 2. As Figure 4 indicates, the leukocyte infiltrate was also reduced in animals treated with PCE (Figure 4C) as compared to that in those that received only DSS (Figure 4B). This observation is consistent with the decrease in MPO activity levels, an index of neutrophilic infiltration in the mucosa. As shown in Figure 5, after 7 days of exposure to 5% DSS, the mucosal MPO activity increased markedly to a value of 22.37 ± 0.66 U/g of tissue. This MPO activity was reduced by 64% in PCE-treated animals.

Because nitric oxide is both a common marker of inflammation and a mediator involved in the pathogenesis of IBD, we also examined the effect of PCE treatment on nitric oxide secretion. As shown in Figure 6B, colons of animals that received only 5% DSS exhibited a 60-fold increase in nitric oxide production as compared with the colons of animals that received only water. PCE treatment abrogated this response and resulted in nitric oxide levels similar to those observed in blank animals.

To determine the possible mechanism of PCE in DSS-induced colitis, we obtained the cytosolic and nuclear fractions from the affected mouse colons to study the effects of PCE treatment on cyclooxygenase-2, p65, pSTAT-3, and pSTAT1α, four proteins involved in the pathogenesis of ulcerative colitis. A significant reduction of cyclooxygenase-2 expression can be observed in Figure 7, as well as a reduction in pSTAT-3 and pSTAT1α

Table 4. Procyanidin Profile (g/100 g of PCE on a Wet Basis) from Monomers through Polymers of the Polyphenol-Enriched Cocoa Extract[a]

procyanidins[b] (g/100 g of PCE)											
1-mers	2-mers	3-mers	4-mers	5-mers	6-mers	7-mers	8-mers	9-mers	10-mers	>10-mers	total
6.16	4.11	4.80	3.61	2.08	2.10	0.35	0.25	0.43	0.00	2.14	26.02

[a] 1-mers, monomers; 2-mers, dimers; 3-mers, trimers; 4-mers, tetramers; 5-mers, pentamers; 6-mers, hexamers; 7-mers, heptamers; 8-mers, octamers; 9-mers, nonamers; 10-mers, decamers; >10-mers, polymers. [b] Data obtained with normal-phase HPLC.

	Colorectum (mm)	Body Weight (g)		
		0 days	4 days	8 days
Blank	94.17 ± 3.27	20.06 ± 0.41	20.37 ± 0.22	19,99 ± 0.52
DSS	49.17 ± 1.54[a]	19.96 ± 0.74	20.35 ± 0.63	15.75 ± 0.80[d]
DSS + PCE	59.17 ± 2.39[b,c]	18.63 ± 0.19	19.16 ± 0.17	16.98 ± 0.25[e,f]

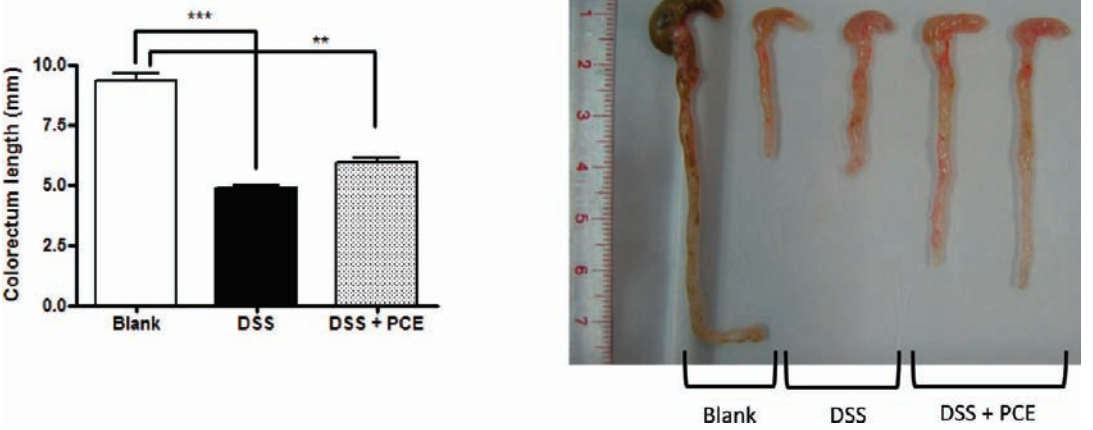

Figure 2. Effect of PCE on weight loss and colon length. In the control group, mice received fresh tap water ad libitum; in the DSS group, mice received fresh tap water with 5% DSS for 7 days. A third group of mice received tap water with 5% DSS and were treated with two 500 mg/kg doses of PCE on days 1 and 4 of the experiment. The weights of the mice were recorded on days 1, 4, and 8. At the end of the experiment, all mice were killed by means of cervical dislocation, and their large intestines were removed. After being washed with ice-cold PBS, the intestines were placed on filter paper and measured without the cecum. Statistical analysis was performed with a nonparametric post hoc test (Kruskal−Wallis test). [a], $P < 0.001$ vs control group; [b], $P < 0.05$ vs control group; [c], $P > 0.05$ vs DSS group; [d], $P < 0.01$ vs control group; [e], $P > 0.05$ vs control group; [f], $P > 0.05$ vs DSS group. The results shown are representative of three independent experiments with 6 to 10 mice per group.

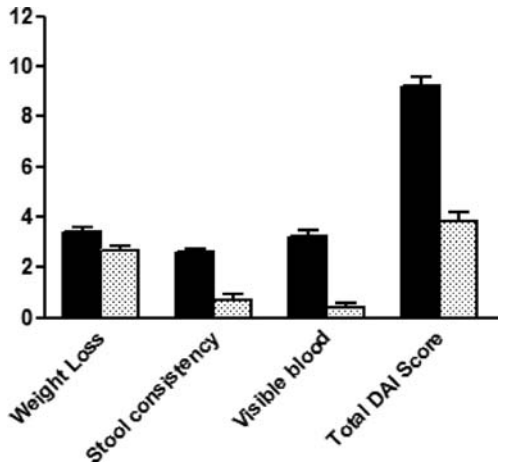

Figure 3. Effect of PCE on the disease activity index (DAI). The DAI was evaluated for DSS groups (black bars) and PCE-treated groups (white bars) upon termination of the experiment in accordance with the scoring system in Table 1. PCE-treated mice showed a reduction in weight loss, normal stool consistency, and slight or no visible blood in their feces, resulting in a lower total DAI score as compared to that of the DSS-group (control).

phosphorylation; however, PCE exerted no effect on NF-κB p65 in the nuclear extract of the mid and distal colon.

Pro-inflammatory mediators play a central role in the pathogenesis of IBD. The observed immune cell infiltration due to enhanced intestinal permeability increases mucosal production of pro-inflammatory cytokines. The overall expression of some of these pro-inflammatory cytokines (IL-6, IL-1β, TNF-α, and IFN-γ) was observed both in mouse peritoneal macrophages and in colon cultures of noncolitic, colitic, and PCE-treated colitic mice. As shown in Figure 8, levels of IL-6, IL-1β, TNF-α, and IFN-γ in peritoneal macrophages increased markedly after stimulation with LPS. We detected a significant decrease in the production of IL-6, IL-1β, and TNF-α (but not of IFN-γ) when those macrophages were treated with PCE 1 h before stimulation (Figure 8). Analysis of these pro-inflammatory enzymes in the colon supernatants revealed that PCE treatment led to a significant reduction of IL-6 (Figure 6A), but not of other cytokines (data not shown).

To further examine the effect of PCE on NF-κB activation in macrophages, we performed an NF-κB reporter gene assay in RAW 264.7 cells (Figure 9). While treatment of the transfected cells with LPS for 18 h increased luciferase activity above basal levels, pretreating cells with PCE (10 μg/mL) inhibited this activity.

DISCUSSION

The use of polyphenols in the treatment of IBD has been reported as a possible preventive treatment, with several studies demonstrating a perceptible delay in the development of the disease.[23] However, this indication for polyphenols is still being questioned because different authors maintain that these compounds are either not absorbed or that they are metabolized in the intestine. However, several studies have provided evidence of the bioavailability of procyanidins, including that published by

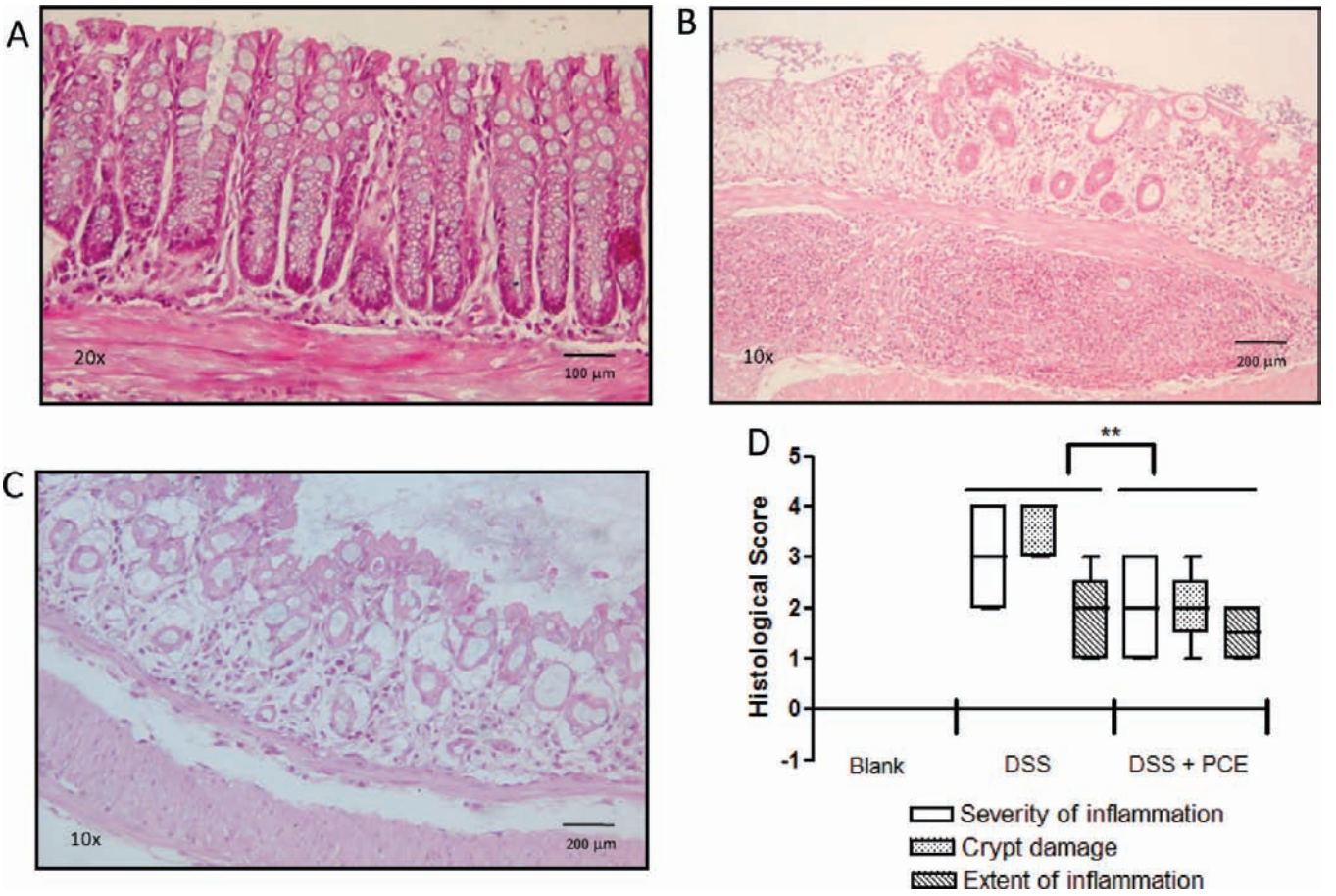

Figure 4. Effect of PCE treatment on histological parameters in acute colitis. Three representative colonic hematoxylin sections were taken from mice receiving fresh tap water (A), fresh tap water with 5% DSS (B), or fresh tap water with DSS 5% and PCE treatment (500 mg/kg) (C) as described in the Materials and Methods section. The severity of inflammation (white bars), extent of inflammation (dotted bars), and crypt damage (lined bars) were determined as described in Table 2. The data presented are representative of three independent experiments with 6 to 10 mice per group. The boundaries of the box indicate the 25th and 75th percentiles; the line within the box marks the median. Whiskers indicate the 90th and 10th percentiles. Differences with the DSS group were determined by means of a one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. **Significantly different from the total histological score of the non-PCE-treated group ($P < 0.01$; Dunnett's *t*-test).

Bombardelli et al.,[24] who fed a ^{14}C-labeled polyphenol product to mice. Bioavailability in humans has also been confirmed by the presence of monomeric flavanols and procyanidin dimers in blood after consumption. Moreover, it has been shown that a significant fraction of larger cocoa procyanidins reaches the upper intestine intact.[25] Finally, the positive effects of cocoa extracts on the cardiovascular system as well as in different experimental protocols of inflammation indicate that the original compounds and/or some of their active metabolites are present in both blood and inflamed tissues.[11,12,26]

Previous studies have described polyphenols from various sources, including green tea, pomegranate, or red wine, but no specific studies on cocoa had been developed until now. The fact that recent research has associated health effects with the consumption of cocoa flavonoids, however, has increased the interest in obtaining products with high cocoa polyphenol content.[10,27,28]

It seems that the mechanism of action of polyphenols stems not only from their known antioxidant properties but also from their ability to interfere with various mediators and transcription factors.[23,28] While it is true that an array of mediators and transcription factors are implicated in the pathogenesis of UC, special attention must go to the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, as well as to the transcription factors NF-κB,

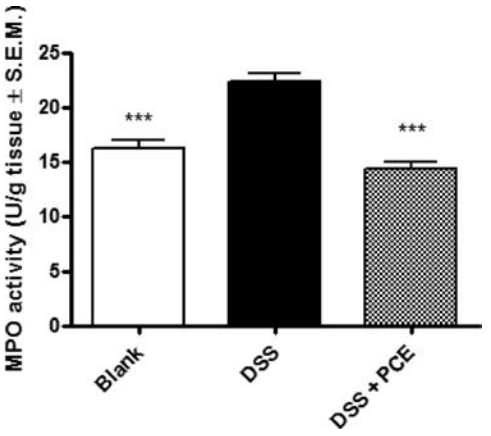

Figure 5. Effect of PCE on MPO activity. Data are expressed as the mean ± SEM. Differences with the DSS group were determined by means of a one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. ***Significantly different from the DSS group ($P < 0.01$; Dunnett's *t*-test; $n = 8$).

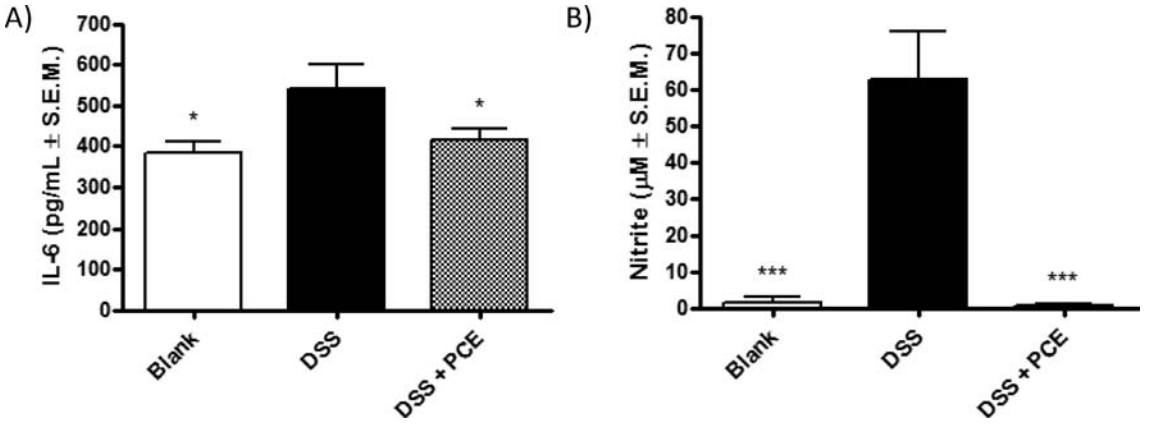

Figure 6. IL-6 secretion and nitric oxide production in colon cultures. Colons were cultured for 24 h, supernatants were collected, and IL-6 was measured in duplicate with ELISA. Nitric oxide production was also measured in duplicate with the aid of the Griess reagent. Results are expressed as IL-6 pg/mL ± SEM and nitrite μM ± SEM, respectively, and are representative of three independent experiments with 2 colons per group. Differences with the DSS group were determined by means of a one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. *Significantly different from the DSS group (*$P < 0.05$; ***$P < 0.01$; Dunnett's *t*-test).

STAT1, and STAT3. In our study, PCE reduced tissue damage and neutrophil infiltration by lowering both STAT1 and STAT3 phosphorylation levels, but had no significant effect on NF-κB p65 in vivo.

Many immune regulatory genes contain specific binding sites for STAT1 in their promoter regions. Moreover, the activation of STAT1 triggers an important signaling pathway for many cytokine and growth factor receptors. In IBD there is an increased expression and activation of STAT1 in neutrophils and monocytes present in the intestinal *lamina propria*; this activation is greater in UC than in CD.[29] The role of STAT3 in IBD has also been described, both in animal and human studies,[30] which have demonstrated that this transcription factor plays different roles in innate and acquired immune cells, making it a relevant factor in the pathogenesis of IBD. STAT3 can be activated through phosphorylation by different cytokines and growth factors, which causes its dimerization and migration to the nucleus to induce the expression of various genes, including those for pro-inflammatory enzymes and mediators.[31] These inhibitory effects may partly justify the decrease observed in cytokine production and cyclooxygenase-2 induction. However, a clear inhibitory effect on the three nuclear factors studied was found in primary macrophages, manifesting itself in a significant reduction in the production of various pro-inflammatory mediators such as IL-6, IL-1β, and TNF-α, as well as a decrease in cyclooxygenase-2 expression. It is worth noting, however, that IFN-γ was not affected. In addition, a clear reduction of cyclooxygenase-2 was also observed in the tissue homogenate, but this inhibition could be due to the inhibition of the other transcription factors as well as to the decrease in cell infiltration and its effect on inflammatory cells. Inhibition of TNF-α[28] and cyclooxygenase-2[32] by cocoa polyphenols has previously been reported in other experimental models.

For its part, NF-κB also plays a relevant role in the pathogenesis of inflammation. When activated, this transcription factor migrates to the nucleus and induces gene expression, particularly that of genes implicated in the expression of cytokines involved in the inflammatory response, such as TNF-α and IL-1β. Unfortunately, very few studies have examined the role of NF-κB in IBD, but several researchers have described a clear correlation between both the inhibition of this transcription factor and the reduction of cytokine production and the attenuation of IBD development. Some of the inhibitory compounds studied have been phenolic derivatives, such as epigallocatechin gallate from green tea, which was found to decrease the serum markers of inflammation in induced colitis in rats and mice.[23]

Various polyphenol-enriched extracts and isolated phenolics have been described as potential therapeutic agents against IBD, for example, *Salvia miltiorrhiza*, *Scutellaria baicalensis*, pomegranate and grape extracts, as well as green tea polyphenols,[23] and the isolated compounds quercitrin,[33] peonol,[34] and curcumin.[35] Among the latter, the activity of curcumin has been related to the inhibition of NF-κB activation and to the blockage of infiltrating cells such as CD4 and CD8.[35] Quercitrin's activity, however, has been explained by invoking inducible nitric oxide synthase inhibition as a consequence of NF-κB inhibition.[33] For its part, peonol attenuated experimentally induced colitis through the inhibition of TNF-α-induced transcriptional activity of NF-κB and IFN-γ induction of STAT1.[34] Anti-inflammatory therapies using anticytokine receptors such as anti-TNF-α and anti-IL-6 attenuate the development of IBD,[36] while the inhibition of STAT1 activation suppresses the inflammatory response. We can thus hypothesize that PCE alleviates the symptoms of IBD through one of these mechanisms since it has been shown to markedly inhibit IL-6 production and STAT1 activation. IL-1β was also clearly inhibited in mouse macrophages. However, PCE's effects on the other cytokines assayed, notably TNF-α and IFN-γ, were quite modest. This finding is interesting in its own right, especially in light of research conducted by Grivennikov and Karin,[37] who demonstrated that NF-κB and STAT3 are constitutively activated in neoplastic cells and may constitute a dangerous liaison in the development of cancer. The systemic inhibition of both STAT3 and NF-κB probably results in enhanced damage to normal cells and tissues; therefore, partial inhibition of STAT3 without affecting NF-κB could circumvent the potential risks of double inhibition while promoting the benefits of PCE, thus avoiding the development of colon cancer, which is a significant risk in patients suffering IBD.

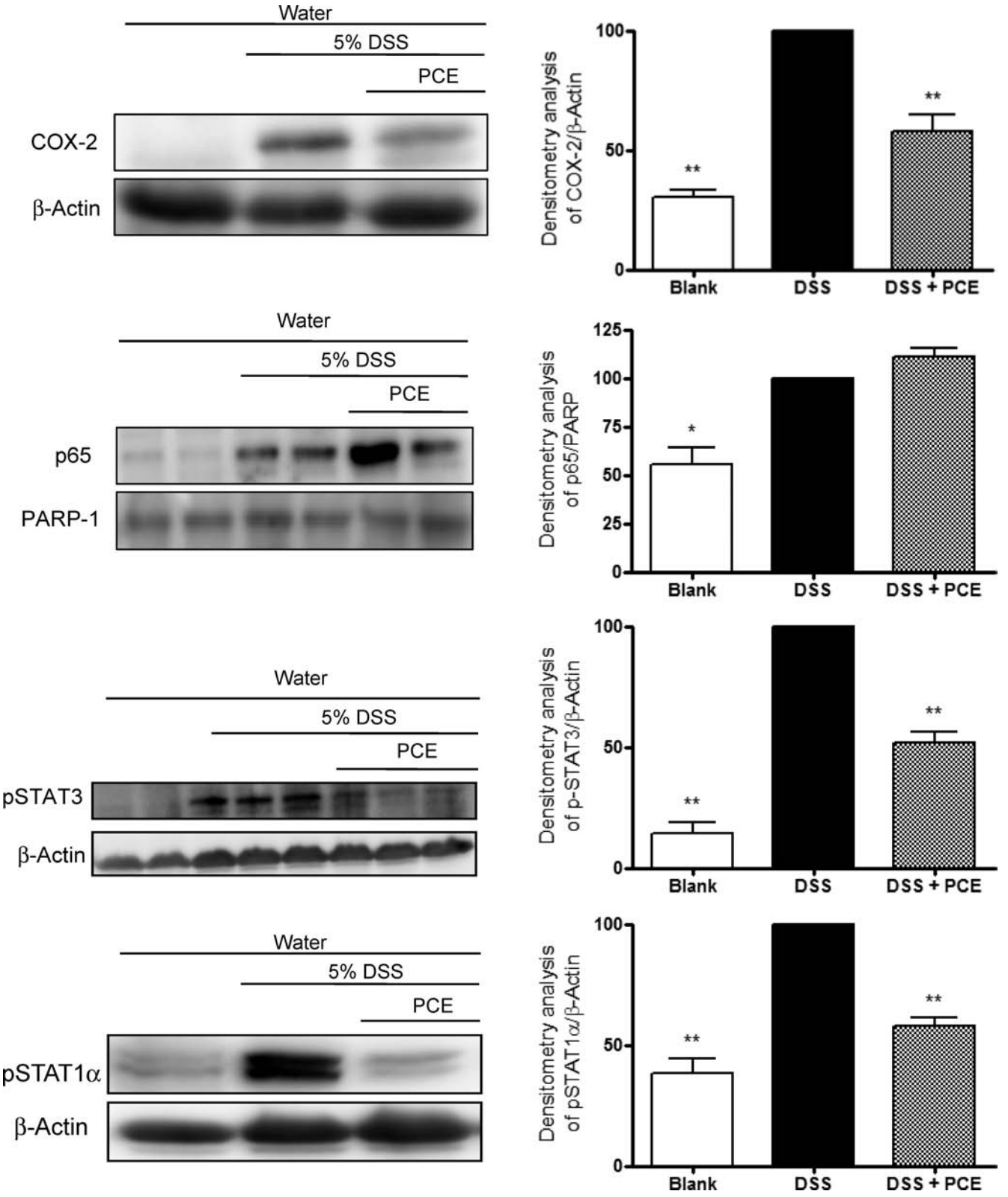

Figure 7. Effects of PCE on cyclooxygenase-2 (COX-2) expression and on nuclear translocation of p65, pSTAT3, and pSTAT1α. The left panels show an example of the Western blot following probing with the corresponding antibody. The histograms to the right represent the data derived from the Western blots following densitometry analysis and considering the DSS group as having 100% expression. Differences with the DSS group were determined by means of a one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. *Significantly different from the DSS group (*$P < 0.05$; **$P < 0.01$; Dunnett's *t*-test; $n = 7-10$).

In conclusion, we have demonstrated the inhibitory effect of PCE on acute UC and elucidated a possible mechanism by which it reduces the physiopathological effect of DSS on the intestine. These findings indicate the utility of PCE and other

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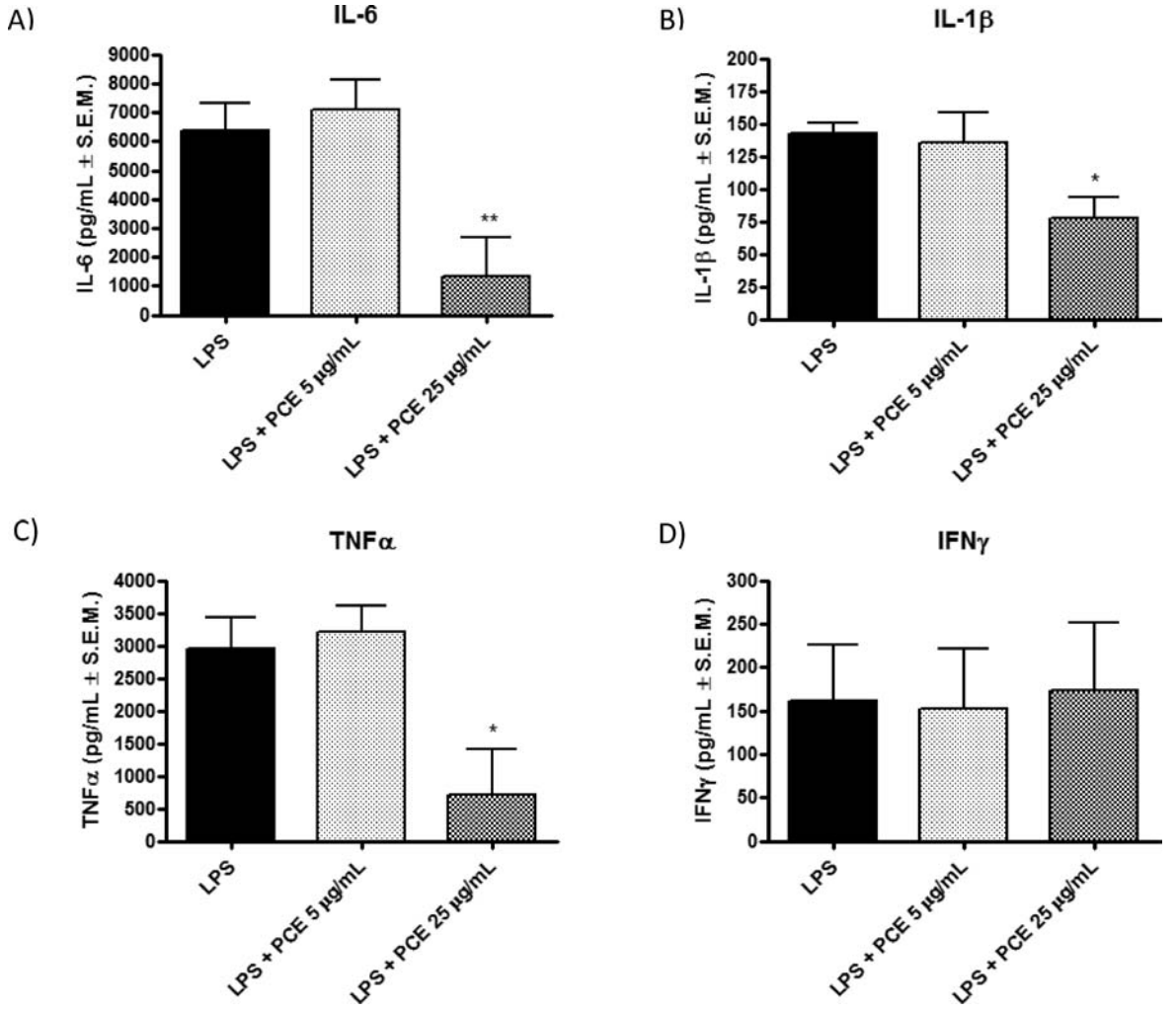

Figure 8. Overall cytokine production in peritoneal macrophages. Mouse peritoneal macrophages were isolated and cultured for 24 h. Supernatants were collected, and cytokines were measured in duplicate with ELISA. The LPS group represents those macrophages stimulated with LPS (1 μg/mL), the second group was treated with PCE (5 μg/mL) prior to stimulation with LPS (1 μg/mL), and the third group represents macrophages stimulated with LPS (1 μg/mL) after prior treatment with PCE (25 μg/mL) for 1 h. Results are expressed as cytokine concentration pg/mL ± SEM and are representative of three independent experiments. Differences with the LPS group were determined by means of a one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test (*$P < 0.05$).

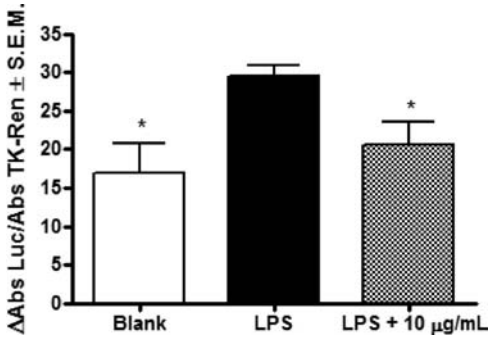

Figure 9. Luciferase assay showing the effect of PCE on lipopolysaccharide (LPS)-induced NF-κB activity in RAW 264.7 macrophages. Luciferase activity was normalized to TK-Renilla activity. The data were obtained from four independent experiments and expressed as the mean (ΔAbs) ± SEM. Differences in absorbance between each group and the control group were determined by means of a one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test (* $P < 0.05$, $n = 4$).

polyphenols from cocoa as potential protective agents against acute UC.

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Notes

Last year, E. Cienfuegos-Jovellanos, S. Laghi, and B. Muguerza worked for Natraceutical. As of the time of writing this article, none of them was still working at said company.

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