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Apple Peel Polyphenols Protect against Gastrointestinal Mucosa Alterations Induced by Indomethacin in Rats

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ABSTRACT: The stability of an apple peel polyphenol extract (APPE) with powerful antioxidant activity was evaluated under acidic conditions in vitro, and its protective effect against gastrointestinal damage was investigated in rats treated with indomethacin. The antioxidant activity of APPE remained stable at pH 2.0 for 4 h. In rats treated with indomethacin (40 mg/kg ig), the previous administration of APPE protected the gastric, intestinal, and colonic mucosa from oxidative stress by preventing increased malondialdehyde concentrations and decreasing the GSH/GSSG ratio. APPE also displayed anti-inflammatory effects by preventing neutrophil infiltration in the mucosa, as evidenced by the lower myeloperoxidase activity. These protective effects of APPE resulted in the prevention of macro- and microscopic damage and of barrier dysfunction along the gastrointestinal tract of the indomethacin-treated animals. This study supports the concept that apple peel polyphenols may be useful in the prevention and/or treatment of nonsteroidal anti-inflammatory drug-associated side effects.

KEYWORDS: apple peel polyphenols, gastrointestinal damage, gastrointestinal permeability, oxidative stress

INTRODUCTION

The polyphenols are a group of secondary metabolites widely expressed in fruits and vegetables. These bioactive compounds have received considerable interest during the past decade due to their wide range of biological activities and their possible role in the prevention of chronic diseases.[1] Apples (*Malus* spp., Rosaceae) represent an important source of polyphenols in the Western diet, and it has been suggested that their regular intake may result in health benefits by reducing the risk of cardiovascular disease, pulmonary dysfunction, diabetes, obesity, cancer, and inflammatory disorders.[1]

Using the worldwide consumed Granny Smith variety, we recently prepared an apple peel polyphenol-rich extract (APPE), which contained 100 times more polyphenols than the whole fruit. The polyphenolic composition of APPE shows a profile identical to that of the fresh peel used for its preparation.[2,3] APPE was shown to inhibit the respiratory burst of neutrophils stimulated by *Helicobacter pylori*, a pathogen that specifically colonizes the human gastric mucosa, and to exert an inhibitory effect both in vitro and in vivo against this microorganism.[3] APPE was also reported to exhibit an anti-inflammatory effect on *H. pylori*-associated gastritis, lowering malondialdehyde levels and gastritis scores.[4] In addition, we recently showed that it exerts a powerful antioxidant effect and protects intestinal Caco-2 cells against the oxidative mitochondrial and cell damage induced by indomethacin (INDO), a nonsteroidal anti-inflammatory drug (NSAID).[5]

NSAIDs are widely used in the treatment of inflammatory disorders but, unfortunately, their chronic administration is frequently associated with adverse effects affecting the gastrointestinal (GI) tract.[6] Various mechanisms have been shown to be involved in the development of INDO-associated GI lesions such as the inhibition of cyclooxygenase 1 (COX-1)[6] and the induction of mitochondrial dysfunction[7−12] and oxidative stress. In fact, INDO has been shown to increase both lipid peroxidation and intracellular oxidative status and to decrease the GSH/GSSG ratio in gastric and intestinal cell lines.[5,11] INDO has also been shown to enhance lipid peroxidation[10,12−16] and the production of reactive oxygen species (ROS)[10] in the GI mucosa of laboratory animals. In addition, it has also been reported to enhance the activity of pro-oxidant enzymes such as NADPH oxidase,[13] myeloperoxidase,[14,16,17] and xanthine oxidase (XO)[12,15] and to decrease that of antioxidant enzymes such as catalase,[12,15] superoxide dismutase (SOD),[15] and glutathione peroxidase[12,15] in the GI mucosa. Interestingly, the oxidative stress induced by INDO in rats has also been associated with disturbances of the GI barrier function, an alteration that is considered to be a primary step in ulcer formation.[18]

On the basis of the role of oxidative stress in the genesis of the cytotoxic effects induced by INDO, the gastroprotective effect of a variety of molecules with antioxidant properties such as curcumin, rebamipide, or melatonin has been successfully evaluated in relation with the gastric damage induced by this drug.[11] The effects of dietary polyphenols have also been considered, including a whole apple extract with a polyphenolic profile different from that of APPE, which has been shown to protect rats against

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the gastric injury induced by the subcutaneous administration of INDO[19] or intragastric aspirin.[20] Taking into account the key role of oxidative stress in the development of the GI injury induced by INDO and the protective effects displayed in vitro by APPE, the aim of the present study was to assess in vitro the stability of the APPE polyphenols in acidic conditions and to determine their potential in reducing the oxidative and inflammatory processes leading to the loss of GI barrier function and to damage of the GI mucosa in INDO-treated rats.

MATERIALS AND METHODS

Apple Peel Extract. The APPE was prepared as previously described.[2,3] The total polyphenolic content (TPC) of APPE, determined by the Folin−Ciocalteu method, represented 600 mg of gallic acid equivalents (GAE) per gram of dried extract. Sixty percent of these polyphenols were flavonoids, primarily quercetin glycosides (hyperoside, rutin, isoquercitrin, and quercitrin), 24% were procyanidins, and 5% were flavan-3-ols, mostly epicatechin.[2,3]

Determination of APPE Polyphenol Stability in Acidic Condition. The stability of APPE in conditions of pH similar to that observed in gastric juice was assessed using with minor modifications procedures previously published.[4] APPE (250 mg) was dissolved in 25 mL of a solution containing (0.24% NaCl w/v and HCl, pH ∼2) and incubated for 6 h at 37 °C with stirring (100 rpm). Aliquots of 200 μL were obtained every hour and assessed for TPC and free radical scavenging and antioxidant capacity. Polyphenols from APPE and acid-treated APPE were separated by thin layer chromatography carried out on 20 × 10 cm silica gel HPTLC 60 F254 plates (Merck). Samples were diluted with methanol, spotted as 7 mm bands using an automatic applicator (Linomat V, Camag), and eluted with toluene/acetone/formic acid (3:6:1, v/v).[2] Flavan-3-ols and procyanidins were detected by staining with dimethylaminocinnamaldehyde (DMACA), whereas flavonoid glycosides were detected with 1% $AlCl_3$ in methanol. The percentages of degradation of procyanidins and quercetin glycosides in acid-treated APPE were evaluated by scanning densitometry at 765 nm and fluorescence at 366 nm using a Camag TLC Scanner IV (Camag, Switzerland) controlled by Cats software (version 4.03). Peak areas were determined using quercetin and epicatechin as standards for calibration. Flavonoids were observed as green fluorescent bands (R_f = 0.3−0.95), and any effect on their stability could be evidenced as a decrease in the sum of peak area in this zone and a correlative increase of the area corresponding to the quercetin peak (R_f = 0.95) resulting from glycoside hydrolysis. Similarly, the sum of peak area between 0.1 and 0.90 and corresponding to procyanidins after DMACA staining was compared with the increase of peak area corresponding to epicatechin monomers (R_f = 0.90), resulting from procyanidin hydrolysis.

Determination of the Free Radical and Pro-oxidant Species Scavenging Properties and Antioxidant Capacity of APPE. The free radical scavenging property of APPE and acid-treated APPE was determined through its ability to scavenge peroxyl, $O_2^{\bullet-}$ and $HO^{\bullet}$ radicals[5] and HClO.[21] The scavenging activity against $O_2^{\bullet-}$ anions, generated by the hypoxanthine/XO system, was assessed by monitoring the oxidation of DHE ($470_{Ex}/590_{Em}$).[5] The scavenging property against $HO^{\bullet}$ radicals, generated through an ascorbate-driven Fenton reaction, was assayed by measuring deoxyribose oxidation fluorometrically through TBARS formation ($532_{Ex}/553_{Em}$).[5] The HClO-quenching activity was assayed by measuring the extent of the prevention of the fluorescence loss of 5-aminosalicylic acid ($340_{Ex}/500_{Em}$) induced by HClO.[21] Oxygen radical absorption capacity (ORAC) and ferric-reducing antioxidant potential (FRAP) assays were used as previously described[5] to quantify the antioxidant capacity of APPE and acid-treated APPE. The ORAC assay was applied to measure the ability of the extract to prevent the oxidation of fluorescein ($485_{Ex}/520_{Em}$) in the presence of AAPH-derived peroxyl radicals, using Trolox as standard. FRAP capacity was determined through the reduction of the Fe^{3+}−TPTZ complex by assaying changes in $OD_{593\ nm}$ after 3 min of reaction.[5]

Animals. The study protocol was accepted by the Animal Ethic Committee, and all of the procedures were performed in compliance with the Guidelines for Care and Use of Laboratory Animals at INTA, University of Chile. Sixty male Sprague−Dawley rats (180−220 g) purchased from the Pontifical Catholic University, Santiago, Chile, were housed with a 12 h light/dark schedule and fed a standard rodent chow with ad libitum access to water. The animals were fasted for 20 h before the experiments.

Experimental Design. Sixty animals were divided into five groups according the treatments administered. In groups 1−3, GI damage was induced by intragastric (ig) administration of INDO (40 mg/kg, dissolved in 5% $NaHCO_3$, pH 7.0), whereas groups 4 and 5 received the same volume of solvent vehicle. The aqueous solution of APPE was administered ig 30 min before INDO. Animals in group 2 received 175 mg/kg of APPE, those of groups 3 and 4 received 350 mg/kg, and those groups 1 and 5 received only the vehicle. A preliminary study previously performed in a small group of animals with the highest dose of APPE (350 mg/kg) did not show any signs of toxicity or changes in their behavior during the following 24 h.

Four hours after INDO administration, 30 animals were sacrificed by decapitation, and their stomach, the proximal part of the small intestine (including the duodenum), and part of the ascending colon were immediately removed. The stomach was opened along its greater curvature, the small intestine and colon were opened longitudinally along their antimesenteric borders, and tissues were washed in saline solution at 4 °C. The eventual presence of macroscopic damage in these tissues was registered photographically, and a piece of stomach, intestine, and colon was fixed in Bouin's solution for subsequent histological analysis. The remaining gastric and colonic tissues were homogenized with an Ultraturrax (IKA T18 basic), whereas the intestinal mucosa was scraped with a glass slide and homogenized. In each mucosal homogenate myeloperoxidase activity was quantitated as a marker of neutrophil infiltration. Lipid peroxidation was measured as an index of oxidative damage, and the ratio of levels of reduced and oxidized glutathione were quantitated as major components of the endogenous antioxidant status.

The second group of 30 rats was used to evaluate the integrity of the GI barrier function by carrying out a permeability test. For this purpose, each animal was administered intragastrically 2 mL of an aqueous solution containing 1 g of sucrose, 120 mg of lactulose, 80 mg of mannitol, and 60 mg of sucralose. The rats were then housed in stainless steel metabolic cages with wire bottoms to separate feces from urine. Urine was collected for 24 h in plastic tubes containing 10% thymol in 100 μL of isopropanol and kept at 4 °C throughout the procedure.

Macro- and Microscopic Gastrointestinal Damage. Macroscopic damage was assessed by counting and measuring the extension of all the lesions identified in the stomach, duodenum, and colon. A macroscopic damage score was calculated for each sample, as described by Leite et al.[22] For histological analysis, the tissue samples were embedded in paraffin, and 3 μm thick serial sections were stained with hematoxylin−eosin and examined under light microscopy. An experienced pathologist blinded to the treatments administered to the rats evaluated the following parameters: gastric, duodenal, and colonic damage (alterations of tissue architecture, damage to the surface epithelium, venous congestion, and parameters of inflammation (such as the extent and depth of neutrophil and eosinophil infiltration in the lamina propria), as previously described.[23] Each individual parameter was scored on a 0−3 scale on the basis of its severity or the extent of involvement of the mucosa. The histological index represents the sum of the scores estimated for each individual parameter.

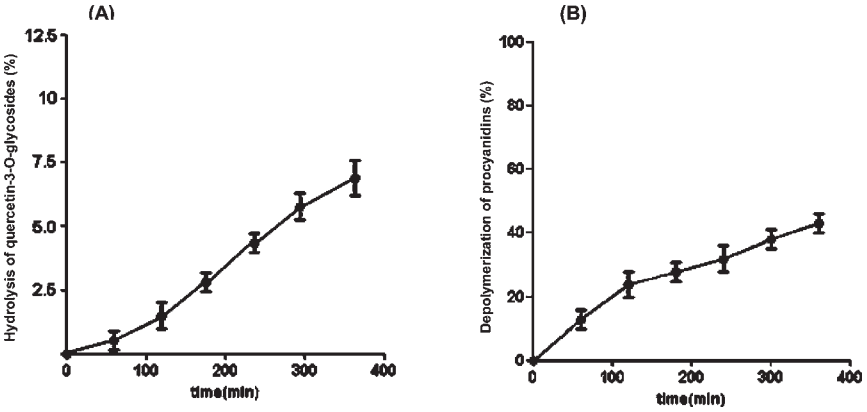

Figure 1. Stability of APPE polyphenols in acidic conditions: (A) hydrolysis of quercetin glycosides, expressed as percentage of quercetin-3-*O*-glycosides degradation; (B) hydrolysis of procyanidins, expressed as percentage of depolymerization of procyanidins. Values represent the mean ± SD, $n = 3$.

Assessment of Myeloperoxidase Activity. Neutrophil infiltration was assessed through the determination of myeloperoxidase activity. The mucosa was homogenized in 50 mM PBS, pH 7.4, and centrifuged at 14000*g* for 10 min at 4 °C. The pellet was homogenized again in 50 mM PBS, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (HETAB) and 10 mM EDTA. This homogenate was subjected to one cycle of freezing−thawing and a brief period of sonication. An aliquot of homogenate was added to a solution containing 80 mM PBS, pH 5.4, 0.5% HETAB, and 1.6 mM 3,3′,5,5′-tetramethylbenzidine (TMB), and the reaction was started by the addition of 0.3 mM H_2O_2. Optical density was read at 655 nm. One unit of myeloperoxidase activity was defined as the amount of enzyme that produced a change in absorbance of 1.0 unit/min at 37 °C.

Determination of Lipid Peroxidation. The determination of the levels of lipid peroxidation in the GI tract was performed by HPLC as previously described.[5] The gastric, intestinal, and colonic mucosas were homogenized in 10 mL of PBS containing 20 mM EDTA. The homogenates were boiled for 60 min with a solution containing 1.22 M metaphosphoric acid and 1% TBA. Malondialdehyde (MDA) was separated using an Agilent Eclipse XDB-C18 (5 μM) column (4.5 × 150 mm), eluted isocratically at 0.6 mL/min with a methanol/25 mM phosphate buffer, pH 6.5 (1:1, v/v), mixture, and detected using a fluorescence detector (wavelengths: excitation, 532 nm; emission, 553 nm). MDA obtained from the acid hydrolysis (40 °C, 60 min) of 1,1,3,3-tetramethoxypropane was used as control standard.

Determination of GSH and GSSG. The glutathione status was assessed according to the method of Hissin and Hilf,[24] based on the reaction of *O*-phthaldehyde (OPT) as a fluorescent reagent with reduced glutathione (GSH) at pH 8.0 and oxidized glutathione (GSSG) at pH 12.0, which involves excitation at 350 nm and fluorescence at 420 nm. For the GSSG estimation, GSH was complexed to *N*-ethylmaleimide (NEM) to prevent interferences of GSH with the measurement of GSSG.

Determination of Urinary Sucrose, Lactulose, Mannitol, and Sucralose. These determinations were carried out as previously described.[25] Urine samples and controls with known amounts of sucrose, lactulose, and mannitol were prepared and analyzed in parallel. Cellobiose and α-methylglucose (Sigma Chemical Co., St. Louis, MO) were used as internal standards. Derivatized sugars were dissolved in 200 μL of hexane, and 2 μL was injected in a Varian 3600 gas chromatograph (Varian Instruments, San Fernando, CA) equipped with a split/splitless injector, a flame ionization detector, and a fused silica capillary column (10 m × 0.32 mm) coated with AT-1701 (Alltech, Deerfield, IL); nitrogen was used as carrier gas. The run-to-run variation of these measurements was <10%. The total amount of sucrose, lactulose, mannitol, and sucralose excreted in the 24 h urine was calculated.

Statistical Analysis. Data were analyzed using GraphPad Prism 4 statistical software. Values represent the means of at least three independent experiments, each conducted in quadruplicate. Microscopic and macroscopic parameters were compared by using nonparametric ANOVA and Dunn's multiple-comparison test, and the other data were analyzed by ANOVA and Tukey's multiple-comparison test.

RESULTS

Stability of APPE Polyphenols in Acidic Medium. We characterized the stability of APPE in terms of its polyphenol content after 6 h of treatment in acidic medium. As observed in Figure 1, the TPC of APPE was not affected by acid treatment during the first 4 h. The flavonoids were mostly quercetin glycosides, and free quercetin was almost undetectable in APPE.[2,3] Free quercetin became detectable only after 4 h of incubation in acidic medium (Figure 1A); the percentage of hydrolysis was 4.2% at 4 h and reached 6.5% after 6 h. The content of procyanidins in APPE decreased in the acidic medium (Figure 1B), and procyanidin hydrolysis reached 27% after 4 h of incubation. However, it is important to note that the exposure of APPE to acidic medium for 4 h did not affect its free radical and pro-oxidant species scavenging or its antioxidant capacity (Table 1).

Effects of APPE on the Macroscopic and Histological Damage Induced by INDO in the GI Mucosa. As shown in Figure 2, the administration of INDO induced an intense mucosal injury that was detectable both macroscopically and histologically. Although the damage was manifested along the entire GI tract, including the duodenal and colonic mucosa, about 90% occurred in the gastric mucosa. Treatment with APPE decreased concentration-dependently the macroscopic damage to the gastric mucosa induced by INDO (Figure 2A), reducing it by 50% at a dose of 175 mg/kg and abolishing it at a dose of 350 mg/kg. In addition, the histological analysis showed that when the animals were treated with APPE, the morphology of their gastric mucosa was comparable to that of the controls, indicating that APPE totally protects the mucosa against the INDO-induced injury (Figure 2B).

INDO caused superficial ulcers with necrotic damage to the gastric glands, surface epithelium disorganization, mucosal infiltration by polymorphonuclear cells, and dilatation of capillaries and venules (Table 2). Rats pretreated with APPE (175 mg/kg) before INDO administration had minimal gastric injuries if any at

Table 1. Free Radical and Pro-oxidant Species Scavenging Properties and Ferric-Reducing Capacity of APPE[a]

	scavenging capacity			ferric-reducing capacity	
exposure to acidic medium (h)	peroxyl radicals (mmol of TE/g of GAE)	superoxide radicals (% of protection against DHE oxidation)	hydroxyl radicals (% of protection against deoxyribose oxidation)	HClO (% of protection against 5-ASA oxidation)	μmol of $FeSO_4$/mg of GAE
1	12 ± 2.2 a	60 ± 1.8 a	23 ± 4.5 a	87 ± 6.5 a	7.8 ± 0.7 a
2	12 ± 1.5 a	62 ± 3.7 a	22 ± 3.4 a	86 ± 2.3 a	7.9 ± 0.3 a
3	12 ± 1.7 a	61 ± 2.3 a	21 ± 4.9 a	84 ± 5.6 a	7.8 ± 0.3 a
4	11 ± 3.0 a	62 ± 4.0 a	20 ± 2.4 a	87 ± 3.6 a	7.9 ± 0.5 a

[a] The ability of APPE to scavenge peroxyl radicals was assessed through the ORAC assay. Results are expressed as millimoles of TE (Trolox equivalents) per gram of GAE. The ability of APPE to scavenge $O_2^{\bullet -}$ and $HO^{\bullet}$ radicals or HClO species was assessed through DHE, deoxyribose, and 5-ASA oxidation, respectively. Results are expressed as percentage of protection against DHE, deoxyribose, or 5-ASA oxidation. Values from assaying the ferric-reducing capacity of APPE represent micromoles of Fe^{2+} equivalents generated per milligram of GAE. Values represent the mean ± SD, $n = 3$. Values bearing different letters are significantly different ($p < 0.05$).

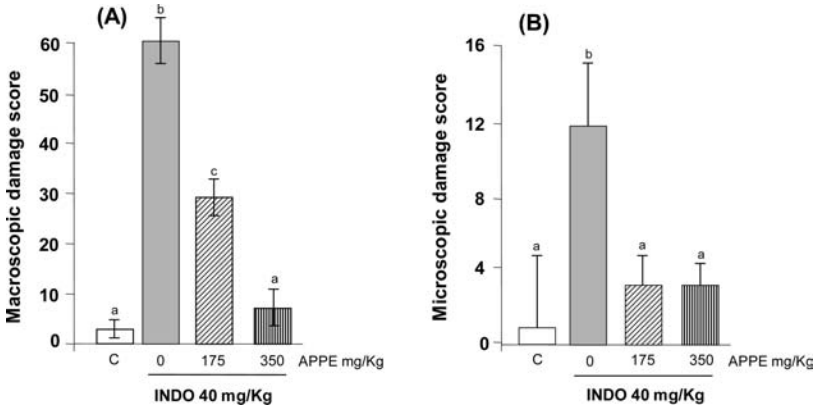

Figure 2. Effect of INDO and APPE on the macro- (A) and microscopic (B) gastric mucosa analysis. Values bearing different letters are significantly different ($p < 0.05$). Values represent the median ± interquartile range, $n = 6$.

Table 2. Effect of INDO and APPE on the Histological Features of the GI Mucosa[a]

histological feature	control	INDO	INDO + APPE 175 mg/kg	INDO + APPE 350 mg/kg
alteration of tissue architecture	0 ± 0 a	1 ± 1 b	0 ± 0 a	0 ± 0 a
damage to the surface epithelium	0.5 ± 0.5 b	2 ± 0.5 b	0 ± 1 a	0 ± 0.5 a
venous congestion	0 ± 0 a	1.5 ± 0.5 b	0 ± 0 a	0 ± 0 a
extent of epithelium damage	0.5 ± 0.5 b	1.5 ± 1.5 b	0 ± 0 a	0 ± 0.5 a
neutrophil and eosinophil infiltration	0 ± 3 a	6 ± 0.5 b	3 ± 1 a	3 ± 0.5 a
	1 ± 4 a	12 ± 3 b	3 ± 2 a	3 ± 1.5 a

[a] Values bearing different letters are significantly different ($p < 0.05$). Values represent the median ± interquartile range, $n = 6$.

all (Table 2). APPE 350 mg/kg (without INDO) had no effect on the mucosal macroscopic and microscopic appearances in the control animals (data not shown).

Effect of APPE on the Myeloperoxidase Activity in the GI Mucosa of INDO-Treated Rats. Oral administration of INDO increased the myeloperoxidase activity in the gastric, duodenal, and colonic mucosa by 105, 67, and 27%, respectively (Figure 3). This increase was dose-dependently prevented by APPE in all the tissues studied. The higher dose of APPE studied, 350 mg/kg, totally prevented the increase in myeloperoxidase activity induced by INDO. APPE 350 mg/kg (without INDO) had no effect on myeloperoxidase activity in the GI mucosa of control animals.

Effect of APPE on the Lipid Peroxidation Activity in the GI Mucosa of INDO-Treated Rats. Compared to the controls, INDO significantly increased lipid peroxidation in the gastric, duodenal, and colonic mucosa by 133, 71, and 80%, respectively. When rats were pretreated with APPE, lipid peroxidation was prevented in a dose-dependent manner in all of the tissues studied (Figure 4). The highest dose of APPE, 350 mg/kg, totally prevented the increase in lipid peroxidation induced by INDO. APPE alone at a dose of 350 mg/kg had no effect on lipid peroxidation in the GI mucosa of control animals.

Effect of APPE on the Levels of Reduced and Oxidized Glutathione Activity in the GI Mucosa of INDO-Treated Rats. INDO reduced the GSH/GSSG ratio by nearly 43% in the

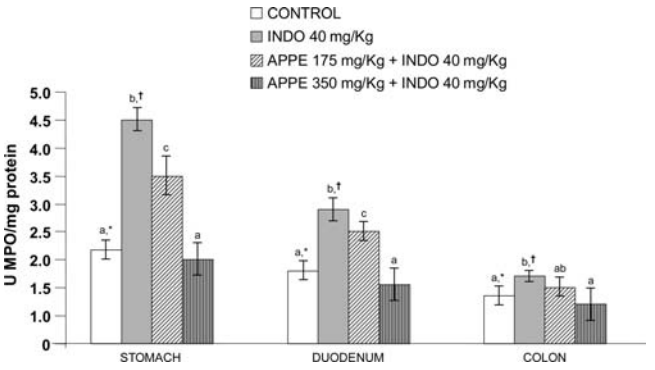

Figure 3. Effect of INDO and APPE on the myeloperoxidase activity of the gastric, duodenal, and colonic mucosa. MPO activity was measured in mucosal homogenates of rats treated with INDO and APPE + INDO. Results are expressed as units of myeloperoxidase (MPO) per milligram of protein. Values bearing different letters are significantly different ($p < 0.05$); this analysis comprises different treatments between the same tissues. The symbols ∗ and † indicate significance ($p < 0.05$) for the difference between values obtained in gastric, duodenal, and colonic mucosa. Values represent the mean ± SD, $n = 6$.

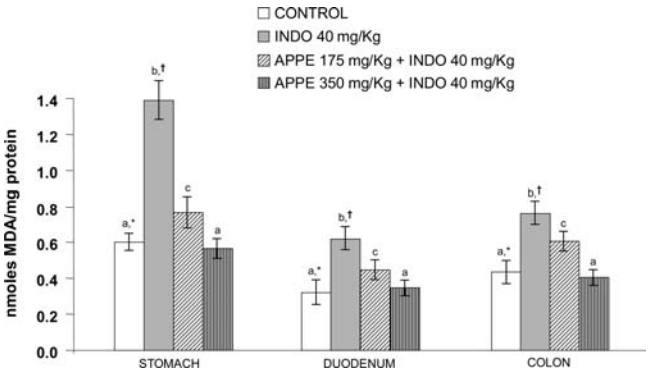

Figure 4. Effect of INDO and APPE on the lipid peroxidation of the gastric, duodenal, and colonic mucosa. MDA levels were measured in mucosal homogenates of rats treated with INDO and APPE + INDO. Results are expressed as nanomoles of MDA per milligram of protein. Values bearing different letters are significantly different ($p < 0.05$); this analysis comprises different treatments between the same tissues. The symbols ∗ and † indicate significance ($p < 0.05$) for the difference between values obtained in gastric, duodenal, and colonic mucosa. Values represent the mean ± SD, $n = 6$.

gastric, duodenal, and colonic mucosa (Figure 5) compared with the control rats. Pretreatment with APPE prevented in a dose-dependent manner the decrease of GSH levels. APPE alone at a dose of 350 mg/kg did not affect the levels of GSH and GSSG in the mucosa of control animals.

Effect of APPE on the GI Barrier Function of INDO-Treated Rats. The GI barrier function was assessed using sucrose lactulose/manitol and sucralose to determine gastric, intestinal, and whole GI tract (including colon) permeability, respectively. As show in Figure 6, INDO increased sucrose permeability by 200% (Figure 6A), the lactulose/manitol ratio by 112% (Figure 6B), and sucralose permeability by 160% (Figure 6C), compared to the control rats. The increase of the lactulose/mannitol ratio was due to the significantly higher excretion of lactulose ($p < 0.001$; data not shown), whereas the excretion of mannitol was not affected. These disturbances of the GI barrier

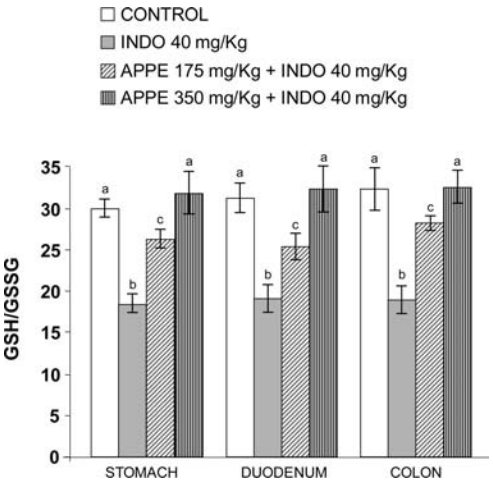

Figure 5. Effect of INDO and APPE on the GSH/GSSG ratio of the gastric, duodenal, and colonic mucosa. GSH and GSSG nanomoles per milligram of protein were measured in mucosal homogenates of rats treated with INDO and APPE + INDO, and the results are expressed as GSH/GSSG. Values bearing different letters are significantly different ($p < 0.05$); this analysis comprises different treatments between the same tissues. The symbols ∗ and † indicate significance ($p < 0.05$) for the difference between values obtained in gastric, duodenal, and colonic mucosa. Values represent the mean ± SD, $n = 6$.

function were totally prevented by pretreatment with APPE 175 or 350 mg/kg. The administration of APPE 350 mg/kg (without INDO) did not affect the GI barrier function of control animals.

DISCUSSION

Oxidative stress is one of the triggering events associated with the GI toxicity of INDO. Although INDO exerts a direct pro-oxidant effect on the GI mucosa,[5,11] animal studies indicate that the oxidative stress induced by this drug could also be secondary to the inflammatory response that typically accompanies tissue damage.[14,16,17] Taking into account these antecedents, the aim of this study was to determine whether the alterations induced by INDO in the GI tract of rats could be prevented by the administration of APPE, a polyphenolic extract from Granny Smith apple peel with powerful antioxidant activity.[5] Our results show that intragastric INDO exerts pro-oxidant effects as reflected by high lipid peroxidation and lower GSH/GSSG ratios, as well as pro-inflammatory effects, as indicated by the increase of myeloperoxidase activity, a marker of mucosal neutrophil infiltration, mainly in the stomach, but also in the duodenum and colon, confirming results obtained in previous studies with similar doses of INDO.[12,14−17] To establish the potential of APPE as a GI protective agent against the damage induced by INDO, we first determined its stability at pH values corresponding to those of gastric juice, specifically in terms of its free radical scavenging and antioxidant capacity. Considering that flavonoids in APPE are mostly quercetin glycosides, results obtained by HPTLC indicate that these compounds were little affected when incubated for 4 h at acidic pH (4.5% of hydrolysis). These results confirm those from Goh and Barlow obtained with flavonol glycosides of *Gingko biloba*[26] and suggest that APPE flavonoid glycosides remain stable in the stomach. In addition, the scavenging capacity of APPE toward $O_2^{\bullet-}$, $HO^{\bullet}$, and HClO and its antioxidant capacity is fully preserved after exposure to acidic medium for 4 h, suggesting that the properties of APPE are not affected during its

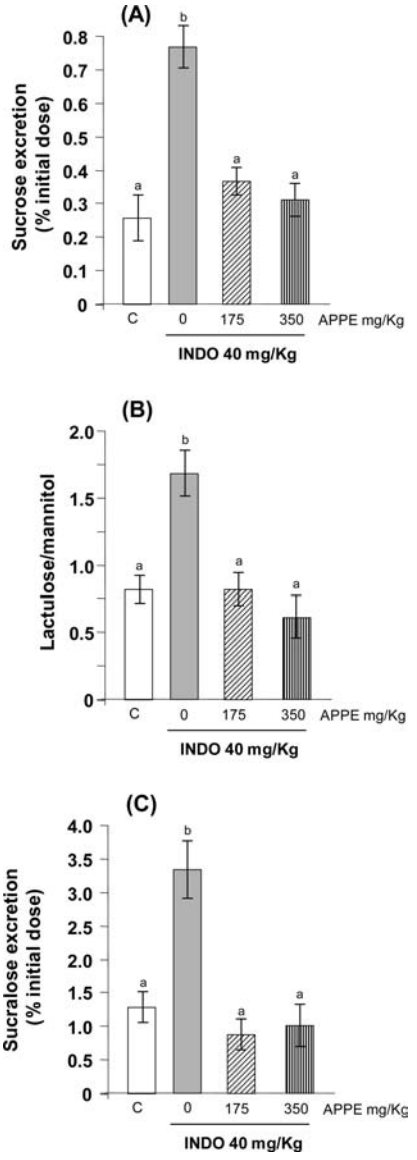

Figure 6. Effect of INDO and APPE on the gastrointestinal barrier function. Sucrose, lactulose, manitol, and sucralose were measured in the urine of rats treated with INDO and APPE + INDO. Results are expressed as percentage of (A) the initial dose of sucrose and (C) sucralose excretion, and as (B) lactulose/manitol. Values bearing different letters are significantly different ($p < 0.05$). Values represent the mean ± SD, $n = 6$.

passing through the stomach. On the other hand, it is important to point out that rutin (quercetin-3-*O*-rhamnoglucoside) was not significantly hydrolyzed in the acidic medium, because this compound is more efficient than quercetin in trapping reactive species such as HOCl, a potent oxidant generated by neutrophils through their myeloperoxidase activity, and capable of chlorinating organic substrates causing significant cell damage. The presence of glycoside in rutin favors its halogenation, a mechanism that prevents the chlorination and subsequent damage of biological molecules.[27]

GSH is an important component of detoxification systems, and it is present in high concentrations in the gastric mucosa. Its depletion after NSAID administration results in higher levels of lipid peroxidation and in the development of erosions and ulcers.[28] Contrarily, increasing GSH levels in the gastric mucosa results in improved protection.[28] In this study the decrease in the GSH/GSSG ratio induced by INDO in the GI mucosa was accompanied by an increase in lipid peroxidation and morphological alterations of the gastric mucosa with ulcer formation. The gastroprotective effect of APPE may be due to its ability to scavenge free radicals and maintain high levels of sulfhydryl group bioavailable (e.g., GSH) in the mucosa, leading to a decrease of the gastric oxidative injury induced by INDO.

On the other hand, we also observed that INDO administration strongly affects the integrity of the GI barrier function, increasing the urinary excretion of sucrose and sucralose as well as the lactulose/manitol ratio, suggesting an alteration of barrier function along the whole GI tract, including the colon. We previously showed that INDO administration increased sucrose urinary excretion as well as the lactulose/manitol ratio in human volunteers, suggesting an increase of their gastric and intestinal permeability.[29] In the present study the oxidative, inflammatory, and morphological alterations induced by INDO were detected mainly in the gastric mucosa, and the more severe changes observed in GI barrier function alterations also occurred at this level: the gastric permeability to sucrose in animals receiving INDO was 3 times that observed in the controls, whereas their lactulose/mannitol ratio was only twice that of the controls. These results confirm other studies showing that INDO administration increased GI permeability to ^{51}Cr-EDTA,[8,18,22] sucrose,[8] and lactulose, mannitol, and sucralose[25] in rats. The increase of lactulose/manitol ratio observed in our study was mainly due to the higher lactulose excretion, suggesting that INDO affects paracellular permeability that is mainly determined by tight-junction permeability, which is regulated by ATP-dependent actin−myosine complexes.[30] This ability of AINEs to alter mitochondrial function and decrease intracellular ATP levels[5,7] may be an underlying mechanism involved in the GI barrier dysfunction induced by INDO. Interestingly, our results show that APPE, which was shown to protect against mitochondrial dysfunction and oxidative stress in vitro,[5] was also capable of preventing the increase in GI permeability induced by INDO in vivo.

It has been postulated that the increased epithelial permeability may expose the GI mucosa to aggressive factors present in the lumen that could stimulate the development of inflammatory responses.[30] In fact, the increase in INDO-induced GI permeability observed in our study was accompanied by an increase in neutrophil infiltration into the gastric mucosa as reflected by the increase in myeloperoxidase activity. Neutrophil infiltration promotes tissue inflammation characterized by ROS overproduction,[7] a phenomenon that was observed in the present study, as suggested by the increase in oxidative parameters in the GI mucosa. Finally, it has been reported that oxidative stress may lead to the development of erosions and ulcers.[8,22] Our results showed that INDO administration induced oxidative stress and alterations of gastric mucosa integrity leading to the formation of ulcers. APPE displayed an anti-inflammatory effect by preventing neutrophil infiltration.

Several strategies have been developed to decrease the risk of GI complications associated with NSAID administration, including administration of H_2-receptor antagonists, proton pump inhibitors, prostaglandin analogues, or selective cyclooxygenase-2 inhibitors. Such strategies have been mainly unsuccessful due to their limited clinical effectiveness and/or to the occurrence of other side effects (e.g., cardiovascular). Accordingly, dietary

polyphenols could represent an interesting and safe alternative as protective agents against the GI mucosal damage induced by NSAIDs. Polyphenols from extra virgin olive oil-enriched diets[14] or from *Mangifera indica* flower decoctions[31] as well as single polyphenols such as quercetin[32] or rutin[16] have been shown to prevent the gastric damage induced by INDO[14,16,32] or piroxicam,[31,32] in rats. Furthermore, in other studies the administration of a polyphenolic extract from whole apple also protected against the ulcerogenic effect induced by the subcutaneous administration of INDO (35 mg/kg)[19] or the oral administration of aspirin.[20] It is important to note that APPE highly differs from the extract used by these authors; the main phenolic compounds present in their extract (% w/w of fresh apple) were catechin (0.012%), chlorogenic acid (0.009%), epicatechin (0.0063%), caffeic acid (0.00018%), rutin (0.00015%), and phloridizin (0.0011%). However, its total polyphenolic content was not determined, and it could only be inferred that it represents about 0.029%; in addition, the main components of the extract are unknown. In the present study, APPE was much more concentrated in polyphenols (about 60%), and its contents in sugars, organic acids, fibers, and minerals were low.[2,3] Futhermore, the polyphenolic profile of APPE completely differed from that used by Graziani et al.[19] and D'Argenio et al.[20] In fact, in addition to the polyphenols described by these authors in their extract, APPE also contains high amounts of quercetin glycosides (60%) and procyanidins (24%). Because quercetin[32] and procyanidins[33] have been previously shown to protect rats against the gastric mucosal damage induced by INDO[32] or acetic acid,[33] their presence in APPE may explain its protective effect. This is also supported by the fact that quercetin is a polyphenol with one of the highest antioxidant and free radical scavenging capacities.[5] It is important to note that our extract protected not only against the oxidative, inflammatory, and macro- and microscopic damage induced by INDO in the animals but also against the alterations of the GI barrier function; this latter aspect was not addressed by Graziani et al.[19] and D'Argenio et al.[20]

In conclusion, these results suggest that APPE protected against the oxidative, inflammatory, and morphological and barrier function alterations induced by INDO in the GI tract. The present study supports the development of clinical studies to evaluate the use of APPE as a nutraceutical to decrease the risk of GI side effects in subjects consuming NSAIDs. More studies are also necessary to evaluate APPE safety in both animal models and humans.

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