Protective Effect of Boldine in Experimental Colitis

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Abstract: The cytoprotective and anti-inflammatory effects of boldine in an experimental model of acute colitis are reported. The administration of boldine to animals with colitis induced by the intrarectal administration of acetic acid, was found to protect against colonic damage as expressed by major reductions in the extent of cell death, tissue disorganization, and edema. Boldine also reduced the colonic neutrophil infiltration, as measured by the myeloperoxidase activity, but it did not significantly affect tissue lipoperoxides. Boldine was found to preserve the colonic fluid transport, a function otherwise markedly affected in the tissue of acid-treated animals. Results presented here provide experimental evidence supporting new cytoprotective and anti-inflammatory properties of boldine.

Key words: *Peumus boldus*, Mol., Monimiaceae, boldine, anti-inflammatory activity, experimental colitis, inflammatory bowel disease.

Introduction

Boldine ([*S*]-2,9-dihydroxy-1,10-dimethoxyaporphine) is a major alkaloid found in the leaves and bark of boldo (*Peumus boldus* Mol.), a common evergreen tree endemic in Chile (1). Recent studies using *in vivo* and *in vitro* biological systems have demonstrated that boldine is a potent scavenger of hydroxyl, lipid, and alkyl peroxyl radicals (2–4), and that it exerts anti-inflammatory activity in carrageenan-induced inflammation of the rat foot-pad (5). Based on these two properties, it has been suggested that boldine may positively interfere with inflammatory disorders in which free radicals act as major mediators of tissue damage (1).

The oxidative modification of cell components mediated by reactive oxygen metabolites (ROMs) has been proposed as a key event in the etiology and/or progression of several inflammatory diseases (6). In the human gastrointestinal tract, enhanced ROMs generation has been implicated in the tissue injury which takes place in inflammatory bowel disease (IBD) (7). The importance of ROMs generation in these pathologies is strongly suggested by the observation that drugs used in the treatment of IBD, such as sulfasalazine and 5-aminosalicylic acid (5-ASA), act as eficient ROM-scavengers (8). Furthermore, administration of superoxide dismutase, an enzyme with potent antioxidant capacity, has also been shown to attenuate tissue damage associated with Crohn's disease (9).

In the present study the antioxidant and cytoprotective effects of boldine were addressed in an *in vivo* model of colonic inflammation in rats in which damage was induced by the intrarectal administration of acetic acid.

Materials and Methods

Chemicals and animals

Boldine was extracted from the bark of *Peumus boldus* Mol. (Monimiaceae), crystallized repeatedly from chloroform, and isolated as the hydrochloride (10). The alkaloid was chromatographically (TLC) pure and its identity was established by infrared and nuclear magnetic resonance spectroscopy (10). All other chemicals were purchased from Sigma (St Louis, MO, USA). Male Sprague-Dawley rats (200–400 g) were obtained from the Institute of Public Health (Santiago, Chile).

Colitis induction

Rats fasted overnight were lightly anaesthetized with diethyl ether and were administered intrarectally either 1.5 ml of acetic acid (4% v/v, pH 2.3) or a saline (NaCl 0.9% w/v) solution (control group) using a pediatric feeding-tube introduced 3–4 cm. The enema was expelled after 20 seconds of contact with the colonic mucosa by gentle massage of the abdomen. After 24 h, the animals were killed by decapitation. Their colons were excised for macroscopic and histologic examination and tissue was processed for the assay of myeloperoxidase (MPO) and the determination of thiobarbituric acid-reactive substances (TBARS). In a few animals in each group, a colonic loop was prepared in order to evaluate the absorptive/secretory functions of the tissue (see below).

Drug treatment

Thirty minutes before the acetic acid or saline instillation, 1 ml of either a saline, boldine solution (100 mg/kg), or a sonicated suspension of 5-ASA (100 mg/kg), was administered intrarectally to diethyl ether lightly anaesthetized animals. All drug solutions were prepared in 0.9% sodium chloride.

Macroscopic analysis

The colon was rapidly excised, opened along its antimesenteric border, gently rinsed of its luminal contents with cold saline solution, placed on a Petri dish chilled to 4 °C, and immediately examined macroscopically. Any visible morphologic damage was scored on a 0–5 scale as described by Morris et al. (11): with 0 representing no damage; 1, localized hyperemia, but no ulcers; 2, linear ulcers without significant inflammation; 3, linear ulcers with inflammation at one site; 4, two or more sites of ulceration and/or inflammation; 5, two or more major sites of inflammation and ulceration, or one major site of inflammation and ulceration extending over 1 cm or more along the length of the colon. Inflammation was defined as areas of hyperemia and bowel thickening. In some animals, the wet weight-to-length ratio of the colon was estimated in order to evaluate the intensity of the edema. For length measurement, the colon was suspended and subjected to a constant weight (8 g).

Histologic analysis

Representative areas of normal and damaged tissue were obtained from the colon and fixed in Bouin's solution; paraffin-embedded serial sections were stained with hematoxylin and eosin and examined under light microscopy. Each slide was evaluated by an independent, trained observer who assessed the following five parameters: overall colonic architecture, appearance of the epithelium of the surface and crypts, cellularity of the lamina propria, and presence of ulcers. Each individual parameter was scored on a 0–5 scale on the basis of its severity or the extent of involvement of the mucosa. The histologic index represents the sum of the scores estimated for each individual parameter.

Myeloperoxidase assay

MPO activity as an index of the neutrophil infiltration into the colonic mucosa was determined as previously described (12). Colonic tissue was gently scraped at 4 °C, weighed and homogenized in 50 mM phosphate buffered saline (PBS), pH 6.0. The homogenate was centrifuged at 10,000 × g during 15 minutes at 4 °C. The supernatant was discarded and the pellet was homogenized again in PBS containing 0.8 % w/v hexadecyltrimethylamonium bromide, freeze-thawed, and sonicated for 20 seconds. After centrifugation, an aliquot of the supernatant was used to determine MPO activity by monitoring spectrophotometrically at 460 nm the decomposition of H_2O_2, using *o*-dianisidine as hydrogen donor. One unit MPO activity was defined as the variation in OD generated during incubation at 37 °C of one gram of homogenized tissue.

Assay of thiobarbituric acid reactive substances

TBARS concentration, as an index of lipid peroxidation was determined as described by Ohkawa et al. (13). Briefly, 200 µl of homogenate prepared as described above were added to an equal volume of 8 % w/v sodium dodecyl sulphate, 1 ml of 20 % v/v acetic acid solution (adjusted to pH 3.5 with NaOH), and 1.5 ml of 0.5 % w/v thiobarbituric acid. The mixture was heated at 100 °C for 60 min. After cooling, 3 ml of *n*-butanol were added, thoroughly mixed, and centrifuged at 2,500 × g for 5 min. The organic layer was withdrawn and its O.D. read at 532 nm against blanks containing no biological material. The results were expressed as the OD per gram of scraped tissue.

In vivo colonic fluid transport

Rats were anaesthetized intraperitoneally with sodium pentobarbital (50 mg/kg) and kept warm under a lamp throughout the experiment. After laparatomy, the colon was exposed and a proximal occluding ligature was placed 2 cm distal from the ceco-colonic junction. The luminal contents were thoroughly flushed out from the colon with saline kept at 37 °C, and the residual saline was gently emptied through the anus. A second, distal occluding ligature was then placed 1 cm above the rectal plaque, thus delimiting a colonic loop. Two ml of saline were carefully injected into the loop using a 27 gauge needle in the proximal part of the loop and the proximal ligature was closed. The colon was returned to the abdominal cavity and the incision was sutured. Sixty minutes later, the animals were killed by decapitation and the colonic loop was removed and detached from all surrounding mesenteric tissue. Each loop length was carefully measured as already described and weighed both when filled and after being emptied of fluid, to determine the intraluminal volume. Fluid transport was calculated as the difference between the initial and the final luminal volumes. It was expressed as the net volume transported across the mucosa per cm of colon, per hour ($\mu l \cdot cm^{-1} \cdot h^{-1}$). A positive difference represents net absorption while negative values indicate net colonic secretion.

Statistical analysis

Results are presented as means ± SEM. A Kruskal-Wallis analysis was performed to check differences between groups. When this overall analysis showed significant ($p < 0.05$) interaction, the Mann-Whitney U test was used to examine the significance of the differences.

Results

Morphological parameters

The intrarectal administration of 4 % acetic acid to rats induced a marked inflammatory response of the colonic mucosa and submucosa after 24 hours. As shown in Figures **1A** and **B**, in acid-treated animals compared with the saline-treated rats, the macroscopic and histologic lesion scores were 4.3 ± 0.2 versus 0.0 ($p = 0.0001$) and 14.6 ± 0.4 versus 2.2 ± 0.7 ($p = 0.0003$), respectively. The wet weight-to-length ratio of the colons, expressed as mg per cm, was 214 ± 20 in the acid-treated rats and 114 ± 7 ($p = 0.0007$) in saline animals, indicating the occurrence of edema in the former group (Fig. **1C**). Pretreatment with a single dose of boldine afforded substantial and statistically significant protection against macroscopic and histologic injuries caused by acetic acid (1.8 ± 0.2 $p = 0.00002$ and 4.8 ± 1.3, $p = 0.001$; respectively). Boldine also protected against acid-induced edema, as shown by decreased total colon weight (130 ± 6; $p = 0.01$). For comparison, a group of acid-treated animals were also given 5-ASA. This latter compound afforded some degree of macroscopic and histologic protection, 2.9 ± 0.5 and 12.6 ± 1.1, respectively, through a decrease of the extent and severity of the lesions produced by the acid.

Biochemical parameters

As shown in Figure **2A**, 4 % acetic acid dramatically increased MPO activity in the rat colonic tissue (111.1 ± 22.7 in the acid-treated versus 2.8 ± 1.6 U/g of scraping in the saline in-

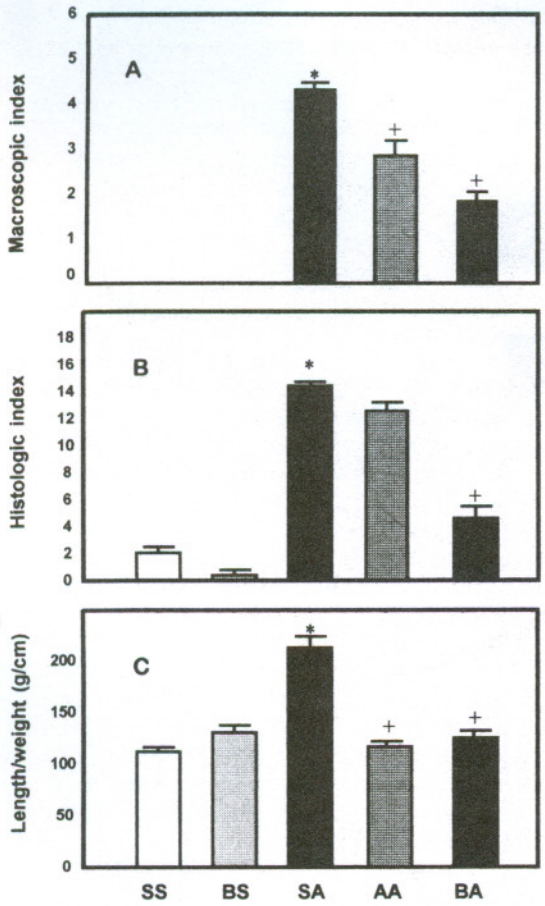

Fig. 1 Macroscopic (**A**), and histologic index (**B**), and presence of edema (**C**). Colitis was experimentally induced in rats by intrarectal administration of 4% acetic acid. Animals were previously treated with saline, 100 mg/kg boldine or 100 mg/kg 5-ASA. Colonic tissues were observed 24 hours after the induction of colitis. SS = Saline/Saline, BS = Boldine/Saline, SA = Saline/Acid, AA = 5-ASA/Acid and BA = Boldine/Acid. Each bar represents mean ± SEM of n ≥ 6 rats. * Statistically significant relative to Saline/Saline (SS) group. + Statistically significant relative to Saline/Acid (SA) group.

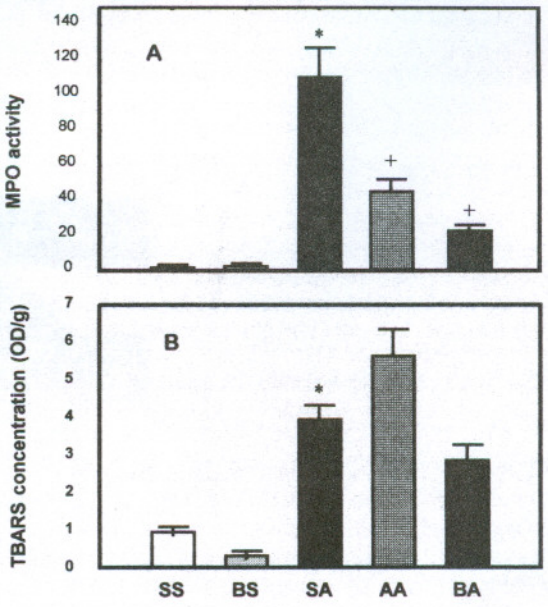

Fig. 2 MPO activity (**A**) and TBARS concentration (**B**) in the mucosal scraping of the colons. Colitis was experimentally induced in rats by intrarectal administration of 4% acetic acid. Animals were previously treated with saline, 100 mg/kg boldine or 100 mg/kg 5-ASA. Colonic tissues were observed 24 hours after the induction of colitis. SS = Saline/Saline, BS = Boldine/Saline, SA = Saline/Acid, AA = 5-ASA/Acid and BA = Boldine/Acid. Each bar represents mean ± SEM of n < 6 rats. * Statistically significant relative to Saline/Saline (SS) group. + Statistically significant relative to Saline/Acid (SA) group.

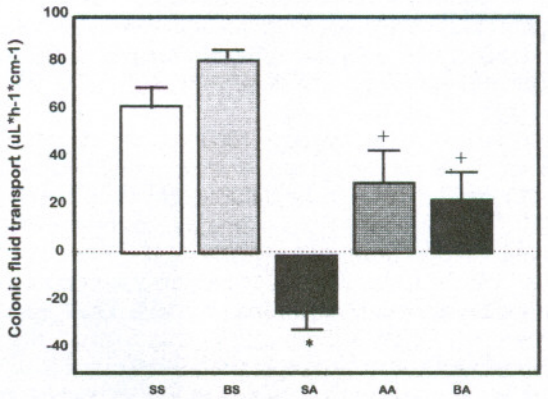

Fig. 3 *In vivo* colonic fluid transport in the saline and acid-treated animals previously administered with saline boldine or 5-ASA. Colonic fluid transport was observed 24 hours after the induction of colitis. A positive value of the transport reflects a net absorption while a negative value indicates a net secretion. SS = Saline/Saline, BS = Boldine/Saline, SA = Saline/Acid, AA = 5-ASA/Acid and BA = Boldine/Acid. Each bar represents mean ± SEM of n ≥ 6 rats. * Statistically significant relative to Saline/Saline (SS) group. + Statistically significant relative to Saline/Acid (SA) group.

fused animals, p = 0.0001). Histological assessment of these tissues confirmed that neutrophils were the predominant cellular infiltrate (data not shown). TBARS concentration in the colonic mucosa (Fig. **2B**) was also significantly increased in the acid-treated animals compared to saline-treated rats (3.98 ± 0.68 versus 0.98 ± 0.27 respectively, p = 0.002), thus suggesting that the acetic acid-induced colitis is associated with the occurrence of lipid peroxidation. Pretreatment with boldine resulted in a substantial and significant reduction of MPO activity (23.5 ± 3.9, p = 0.0007). However, previous administration with boldine prevented only partially and not significantly the increase in TBARS concentration induced by the acid treatment (2.9 ± 0.8, p = 0.25). Previous intrarectal administration of 5-ASA also tended to reduce neutrophil infiltration due to the effect of acetic acid on the mucosa (MPO activity = 46.1 ± 11.2, p = 0.065) but to a lesser extent than boldine while TBARS concentration was slightly but not significantly (5.7 ± 1.1, p = 0.25) higher than in acid-treated rats. It should be noted that in the face of edema almost doubling the wet tissue weight (Fig. **1C**) in the acid-treated animals, the magnitude of the changes in MPO and TBARS in this experimental group (expressed "per g of wet weight") is probably underestimated.

Functional parameter

As shown in Figure **3**, *in vivo* colonic fluid absorption in saline-treated rats was $62.5 \pm 15.0\,\mu l \cdot cm^{-1} \cdot h^{-1}$. Pretreatment of these animals with boldine did not alter colonic fluid absorption. In acetic acid colitis, the net colonic fluid transport became secretory, $-24.9 \pm 13.1\,\mu l \cdot cm^{-1} \cdot h^{-1}$. This degree of alteration in colonic fluid transport is similar to that described by others in this model (14). Pretreatment with boldine or with 5-ASA preserved colonic fluid transport (23.2 ± 22.3 and $30.4 \pm 18.3\,\mu l \cdot cm^{-1} \cdot h^{-1}$, respectively; $p = 0.05$ and $p = 0.02$), although this function had not returned to normal values after 24 hours.

Discussion

Early work by us (1–3) has contributed to establish that boldine is a potent antioxidant capable of protecting biological and non-biological substrates against free radical-mediated oxidative damage. More recently, we reported that, in addition to its free radical-scavenging properties, boldine also has the capacity to act as an effective protector against the cytolytic damage induced to hepatocytes (15) by different xenobiotics whose toxicity is mediated by highly reactive radical species.

Oxygen reactive species are involved in the cyclo- and lipoxygenase-catalyzed conversion of arachidonate into eicosanoid-like inflammatory mediators and are also produced during inflammatory phenomena in association with leucocytes infiltration (16, 17). In consequence, it has been suggested that antioxidant molecules which may interfere with ROMs generation could also have anti-inflammatory effects (18). Along this line, different natural and synthetic antioxidants have been tested and effectively demonstrated to possess anti-inflammatory properties. The present study shows an *in vivo* cytoprotective effect of boldine against acetic acid-induced colonic inflammation. This antioxidant effect affords significant protection to rat colonic mucosa as shown by the improvement of most of the morphological, biochemical, and functional parameters assessed. The results obtained are thus in agreement with the previously observed anti-inflammatory action of boldine in carrageenan-induced guinea pig paw edema (5). It is worth noting that boldine was also shown to directly inhibit *in vitro* prostacyclin synthesis (5). The acetic acid-induced colitis used in this study represents a classical experimental model of human IBD in that it is associated with high tissue levels of MPO activity, and in that it exhibits a pattern of arachidonic acid metabolism strikingly similar to that occurring in human IBD (19). The decrease in MPO activity induced by boldine suggests a lower infiltration of neutrophils in the acid-exposed tissue. Such an effect was concomitantly associated with a considerably lower expression of macroscopic and histologic injury to the colon. Nonetheless, despite its free radical scavenging capacity, boldine failed to prevent cellular lipid peroxidation caused by acetic acid. A possible explanation for this latter fact may be that while boldine largely protects against the macroscopic damage induced by acetic acid, a number of dead colonic cells were still microscopically identifiable after its administration. Since lipid peroxidation may not only be the cause but also the consequence of cell death, the increased TBARS seen after boldine administration could reflect the accumulation of lipoperoxidative products in non-protected, dying cells. Alternatively, it could be argued that while in the present model peroxidation of lipid substrates accompanies cell damage, its prevention by antioxidants like boldine may not be fundamental to achieve cell protection. In addition to lipids, other macromolecules such as proteins may also become the target of free radicals. In fact, the oxidative modification of proteins is increasingly recognized as a significant process leading to cytotoxicity (20, 21). Ongoing work conducted in our laboratory indicates that boldine is highly effective in preventing the oxidative modification associated with the inactivation of the radical-sensitive enzyme lysozime, such an effect was found not to be shared by other recognized chain-breaking antioxidants. In view of its limited effect on lipid peroxidation, it is conceivable that the cytoprotective effects of boldine may relate primarily to the inhibition of the oxidative modification of certain proteins whose free radical-mediated structural modification and loss of function is particularly relevant to the development of cell injury. However, at this point the extent to which protein oxidation is indeed implicated in the acetic acid-induced damage to the colon remains to be established.

On the other hand, the cytoprotective action of boldine seen in this model may also be related to its ability to act as a calcium antagonist (22). In fact, the prior administration of verapamil, a recognized calcium channel blocker, to acid-treated animals results in significant mucosal protection, an effect reported to occur as a consequence of reduced mucosal leukotriene production and increased prostaglandin E_2 synthesis (14). Based on its prostacyclin inhibitory properties, a similar mechanism of action may be proposed for the anti-inflammatory effects of boldine seen in our studies.

In this study we used 5-ASA as a comparative control for boldine in mucosal cytoprotection. It is well known that sulfasalazine and its metabolite 5-aminosalicylic acid are anti-inflammatory agents used in the treatment of Crohn's disease and ulcerative colitis. Our results show that after treatment with 5-ASA, its cytoprotective effect did not exceed that of boldine, and that in the case of lipid peroxidation, a slight increase rather than a decrease, was observed. A reasonable explanation for the cytoprotective activity of 5-ASA may relate to its potent free radical scavenger properties against hypochlorite, superoxide, and hydroxyl radicals (8). It should be kept in mind that all these radicals may be implicated not only in the lipid peroxidative process but also in several inflammatory events which may be taking place in the acetic acid model used here and that are important for the cellular damage they cause (6). In the acetic acid model, lipid peroxidation may be a contributing factor leading to cell damage; however, as was pointed out for boldine, from the results observed with 5-ASA it becames apparent that the prevention of lipid peroxidation by this latter compound would not be essential for cytoprotection. In turn, the effect of 5-ASA on MPO activity suggests that an inhibitory activity on inflammatory mediators would primarily underlie the cytoprotection afforded by this compound.

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