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BRIEF ARTICLE

Protective effects of D-002 on experimentally induced gastroesophageal reflux in rats

Zullyt Zamora, Vivian Molina, Rosa Mas, Yazmin Ravelo, Yohany Perez, Ambar Oyarzabal

Zullyt Zamora, Vivian Molina, Rosa Mas, Yazmin Ravelo, Yohany Perez, Ambar Oyarzabal, Department of Pharmacology, Centre of Natural Products, National Centre for Scientific Research, Havana 10600, Cuba
Author contributions: Zamora Z and Molina V prepared the draft study design, performed the experiments, analyzed the data, and wrote the first version of the manuscript; Mas R provided the original concept of the study, reviewed critically the study design and data analysis, and wrote further versions of the manuscript; Ravelo Y participated in conducting the experiments, data acquisition and analysis, and reviewed the manuscript; Perez Y and Oyarzabal A conducted the biochemical tests and reviewed aspects concerning oxidative variables; all the authors gave final approval of the version of the manuscript to be published.
Correspondence to: Zullyt Zamora, PhD, Department of Pharmacology, Centre of Natural Products, National Centre for Scientific Research, PO Box 6880, Cubanacan, Havana 10600, Cuba. zullyt.zamora@cnic.edu.cu
Telephone: +53-7-2714200 Fax: +53-7-336837
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Abstract

AIM: To investigate the effects of beeswax alcohols (D-002) on the esophageal damage induced by gastroesophageal reflux (GER) in rats.

METHODS: Sixty male rats were randomized into six groups (10 rats/group): a negative control and five groups with experimentally induced GER: a positive vehicle control, three treated with D-002 (25, 100 and 200 mg/kg, respectively), and one with omeprazole 10 mg/kg. All treatments were given by gastric gavage. One hour after dosing, GER was produced by simultaneous ligation of the pyloric end and the forestomach. Esophageal lesions index (ELI), gastric secretion volume and acidity, and esophageal malondialdehyde (MDA) and sulfhydryl (SH) group concentrations were measured. Statistical significance was considered at $P < 0.05$.

RESULTS: As compared to the negative control, the positive control group exhibited increased ELI (5.2 ± 0.33 *vs* 0 ± 0, $P = 0.0003$), gastric secretion volume (2.69 ± 0.09 *vs* 0.1 ± 0.0, $P = 0.0003$) and acidity (238 ± 19.37 *vs* 120.0 ± 5.77, $P = 0.001$), and esophageal concentrations of MDA (2.56 ± 0.1 *vs* 1.76 ± 0.28, $P = 0.001$) and SH groups (1.02 ± 0.05 *vs* 0.56 ± 0.08, $P = 0.0003$). D-002 (25, 100 and 200 mg/kg) reduced ELI (3.36 ± 0.31, 2.90 ± 0.46 and 2.8 ± 0.23, respectively) *vs* the positive control (5.2 ± 0.33) ($P = 0.004$; $P = 0.002$; $P = 0.001$, respectively). There were no significant changes in acidity with D-002 treatment, and only the highest dose reduced the volume of the gastric secretion (1.92 ± 0.25) *vs* the positive control (2.69 ± 0.09, $P = 0.013$). D-002 (25, 100 and 200 mg/kg) lowered the esophageal MDA (2.05 ± 0.16, 1.98 ± 0.22 and 1.93 ± 0.22, respectively) ($P = 0.01$; $P = 0.03$; $P = 0.03$, respectively) and SH group concentration (0.87 ± 0.06, 0.79 ± 0.08 and 0.77 ± 0.06, respectively) ($P = 0.04$; $P = 0.04$; $P = 0.02$) *vs* the positive control (2.56 ± 0.10 and 1.02 ± 0.05, respectively). Omeprazole decreased ELI (2.54 ± 0.47), gastric secretion volume (1.97 ± 0.14) and acidity (158.5 ± 22.79), esophageal MDA (1.87 ± 0.13) and SH group (0.72 ± 0.05) concentrations *vs* the positive control ($P = 0.002$; $P = 0.001$; $P = 0.02$; $P = 0.003$; $P = 0.002$, respectively).

CONCLUSION: Acute oral administration of D-002 decreased macroscopic esophageal lesions and oxidative stress in rats with experimentally induced GER, without modifying gastric secretion acidity.

Key words: D-002; Beeswax alcohols; Esophagitis; Gastroesophageal reflux; Oxidative stress

Core tip: Beeswax alcohols (D-002) has gastroprotective effects in experimental and clinical studies. How-

ever, the effects of D-002 on gastroesophageal reflux (GER) have not been investigated. We demonstrated that acute oral administration of 25, 100 and 200 mg/kg D-002 decreased the esophageal lesion index, and esophageal malondialdehyde and sulfhydryl group concentrations. Only the highest dose of D-002 reduced the gastric secretion volume, but none modified the acidity. D-002 decreased esophageal lesions and esophageal concentrations of lipid peroxidation and protein oxidation markers in rats with experimental GER, without modifying gastric secretion acidity.

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INTRODUCTION

Gastroesophageal reflux disease (GERD), a chronic and relapsing disease that affects 40% of the adult population worldwide, emerges when the gastric acid flows back into the esophagus[1-4]. GERD progression leads to the erosion of the esophageal mucosal epithelium, which is implicated in the development of Barrett's esophagus and the subsequent increased risk of developing esophageal cancer[5,6].

Although the etiology of the abnormal reflux of the gastric contents from the stomach to the esophagus is complex and involves multiple causes, the disease mainly results from weak anti-reflux barriers at the gastroesophageal junction that become incompetent to protect against increased reflux, thus leading to esophageal erosion and inflammation. The imbalance between aggressive (refluxed gastric acid secretion and duodenal juice) and defensive factors (esophageal acid clearance and tissue resistance) contributes to the esophageal damage[7-9]. The acid secretion into the esophagus trigger this process, but when it acts together with small amounts of pepsin it increases the risk of esophageal mucosal damage[10]. Gastroesophageal reflux (GER)-induced increase in inflammatory mediators and reactive oxygen species have been shown to contribute to the mucosal damage[11,12].

Current management of GERD includes the use of antisecretory treatments aimed primarily at reducing gastric acidity, such as the proton pump inhibitors (PPIs) or H_2 receptor antagonists (H_2RAs)[13,14]. In particular, acid suppression achieved with PPIs is the mainstay of therapy for reflux disease, but despite this, symptoms and damage persist and recur in many patients[15].

In terms of safety, PPIs and H_2RAs both have a good safety profile[13,14], but recent data suggest a link between their use and some long-term effects of clinical relevance, of which the increased risk of fractures, mainly in the elderly, seems to most supported by the evidence available[16-18]. The benefits of current therapy to manage GERD, however, outweigh the risks, but the search for new effective and safer treatments is ongoing.

Beeswax alcohols (D-002), a mixture of six higher aliphatic primary alcohols purified from the beeswax, in which traicontanol is the major component[19], has been shown to produce gastroprotective effects through multiple mechanisms that mainly involve increased gastric mucus secretion and improved mucus composition (increased content of mucus proteins, glycoproteins and sulfated macromolecules)[20-22]. In addition, D-002 exhibits antioxidant and anti-inflammatory effects on the gastric mucosa[23,24], which could contribute additionally to the gastroesophageal protection.

Oral administration of D-002 reduces the generation of hydroxyl radicals *in vivo*[24], the concentration of malondialdehyde (MDA) (a lipid peroxidation marker)[23,24] and carbonyl groups (a protein oxidation marker), and myeloperoxidase activity (a marker of inflammation), but increases the activity of glutathione peroxidase, superoxide dismutase and catalase in the gastric mucosa of rats with indomethacin-induced ulcers[24].

In light of these findings, we supposed that D-002 could be beneficial for ameliorating GER, which has not yet been investigated. The present study was undertaken to investigate the effect of D-002 on experimentally induced GER in rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200-250 g) purchased from the National Centre for Laboratory Animals Production (CENPALAB, Havana, Cuba) were adapted for 7 d to the experimental conditions: temperature 25 ℃ ± 2 ℃, humidity 60% ± 5% and light/dark cycles of 12 h. Standard chow pellets from CENPALAB and water were given *ad libitum*. Rats were deprived of food for the 24 h prior to GER induction, but with free access to water.

Animal experiments were conducted in accordance with the Cuban Guidelines of Animals Handling and the Cuban Code of Good Laboratory Practices, which follow international guidelines for the use and care of laboratory animals. The study protocol and animals use were approved prior to the study by the Institutional Animal Ethics Committee.

Chemicals and test substance

Omeprazole was purchased from DOMER (Mexico). The batch of D-002, supplied by the Plants of Natural Products (Havana, Cuba), had the following composition: tetracosanol (7.0%), hexacosanol (11.5%), octacosanol (12.1%), triacontanol (34.8%), dotriacontanol (22.5%) and tetratiacontanol (2.6%). Purity (total content of these alcohols) was 90.0%.

Dosage and administration

D-002 and omeprazole were suspended in 1% acacia

gum/water. Rats were randomized into six groups of 10 rats each: a negative vehicle control and five exposed to GER induction: a positive vehicle control, three treated with D-002 (25, 100 and 200 mg/kg, respectively), and one with 10 mg/kg omeprazole. All treatments (D-002, omeprazole, or vehicle) were given orally by gastric gavage (1 mL/200 g body weight), 1 h prior to GER induction.

The chosen doses of D-002 were those effective in the model of pylorus-ligation-induced gastric ulceration[20], and the omeprazole dose was that reported as effective in a model of GER in rats[25].

Induction of GER

Under pentobarbital anaesthesia (40 mg/kg intraperitoneally), a midline incision was performed on the abdomen and the pylorus and transitional junction between the forestomach and corpus were simultaneously ligated to induce reflux of gastric juice into the esophagus[26]. The abdominal cavity was then closed and 5 h later, rats were sacrificed under anaesthesia. The gastric content was collected and centrifuged at 3000 r/min for 10 min. The volume of the supernatant was measured and its acidity estimated by titration with 0.1 mol/L NaOH to pH = 7.0[20]. The esophagus was removed, incised lengthwise, and then the macroscopic esophageal lesions were observed under a microscope and measured. The esophageal tissue was stored at -20 ℃ until performing the biochemical analyses.

Esophageal lesion index

The esophageal lesion index (ELI) score was calculated (macroscopic degree of injury 0-6) after gross inspection of the esophagus under a magnifying glass (× 3) by two independent blinded observers. The lesions were scored as follows: 0: no visible lesion; 1: some erosion and bleeding; 2: total area of lesions < 15 mm^2; 3: total area of lesions < 30 mm^2; 4: total area of lesions < 40 mm^2; 5: total area of lesions < 45 mm^2; and 6: perforation[27].

Oxidative variables

For the estimation of oxidative variables, the excised esophageal tissue was transferred to ice-cooled test tubes and homogenized in 150 mmol/L Tris-HCl buffer (pH = 7.4) containing 0.25 mol/L sucrose-EDTA (1 g tissue/9 mL buffer) by Ultra-Turrax homogenizer T25 (Germany). The homogenates were centrifuged at 5000 *g* for 10 min at 4 ℃, and the supernatants stored at -80 ℃ until analysis. All the assays were performed in triplicate in an Ultrospec Plus LKB spectrophotometer (Pharmacia LkB Biotechnology; Uppsala, Sweden). Protein concentrations were measured by a modification of the Lowry method[28].

Lipid peroxidation assessment: MDA level, a marker of lipid peroxidation in esophageal homogenates, was measured as thiobarbituric acid reactive species (TBARS)[29] Homogenate aliquots (1 mL) were added to a mixture containing 0.2 mL 8.1% SDS plus 1.5 mL 20% acetic acid solution adjusted to pH = 3.5, 1.5 mL of thiobarbituric acid solution, and 1 mmol/L butylated hydroxytoluene, heated at 95 ℃ for 45 min and cooled. One milliliter of distilled water plus 5 mL *n*-butanol: pyridine (15:1 v/v) mixture was added to the mixture, shaken and centrifuged. The organic layer was used for TBARS determination at 535 nm using freshly diluted MDA bis (dimethyl acetal) as a standard. TBARS concentrations were expressed as nmoL MDA/mg protein.

Protein oxidation assessment: SH groups were measured using the 5'5-dithio-bis (2-nitrobenzoic acid) (DTNB) assay[30]. Homogenate aliquots (200 μL) were treated with 600 μL 20 mmol/L Tris-EDTA buffer (pH = 8.2), 40 μL 10 mmol/L DTNB and 3.16 mL absolute ethanol. This mixture was then incubated to ambient temperature for 20 min and centrifuged at 3000 *g* for 10 min. The optical density of the supernatant was measured at 412 nm, using a 13.6/cm/mol coefficient of absorptivity and SH concentrations were reported in mmol/L.

Statistical analysis

Data were expressed as the means ± SE. Paired comparisons between control and treated groups were done with the nonparametric Mann-Whitney *U* test. The level of statistical significance was set at $\alpha = 0.05$. The analyses were carried out using the Statistic software for Windows (Release 4.2, Stat Soft, United States). Dose-effect relationships were assessed by using dose regression linear analysis on the Primer of Biostatistics program (Stanton A, Glantz; McGraw-Hill, Inc Version 3.01).

RESULTS

Effects on esophageal lesions

Five hours after ligation, the positive group displayed macroscopic lesions quantitatively assessed in term of ELI values that were significantly increased as compared to the negative control group, which did not have visible lesions. By contrast, treatment with D-002 (25-200 mg/kg) and omeprazole (10 mg/kg) ameliorated GER-induced esopagheal injury (Table 1).

Acute oral administration of D-002 (25, 100 and 200 mg/kg) reduced the severity of GER-induced oesophagitis (ELI) by 35.4%, 44.2% and 46.1%, respectively, as compared to the positive control group. Despite the fact that the effects increased slightly with dose, the statistical analysis did not show dose dependence. Omeprazole (10 mg/kg), the reference drug, reduced the ELI significantly by 51.1% as compared to the positive control group. The positive control group also exhibited an increase in the volume and acidity of gastric secretion vs the negative control group. Oral administration of the highest dose of D-002 (200 mg/kg) reduced significantly the volume of gastric secretion, but all doses failed to affect the acidity of the gastric secretion. Omeprazole (10 mg/kg) decreased significantly the volume and acidity of the gastric secretion as compared to the positive control

Table 1 Effects on esophageal lesions, gastric secretion volume and acidity, oxidative variable in rats with gastroesophageal reflux

Groups	Doses (mg/kg)	ELI (mean ± SE)	I	Volume (mL)	I	Acidity (meq/L/100 g)	MDA (nmol/mg protein)	I	SH (mmol)	I
Negative control	-	0 ± 0[c]		0.1 ± 0[c]	--	120 ± 5.77[b]	1.76 ± 0.28[b]		0.56 ± 0.08[b]	--
Positive control	-	5.2 ± 0.33		2.69 ± 0.09	--	238 ± 19.37	2.56 ± 0.10		1.02 ± 0.05	--
D-002	25	3.36 ± 0.31[b]	35.4%	2.63 ± 0.36	2.3%	220 ± 22.57	2.05 ± 0.16[a]	64.0%	0.87 ± 0.06[a]	32.6%
D-002	100	2.90 ± 0.46[b]	44.2%	2.28 ± 0.45	19.0%	210 ± 19.79	1.98 ± 0.22[a]	72.5%	0.79 ± 0.08[b]	50.0%
D-002	200	2.8 ± 0.23[b]	46.1%	1.92 ± 0.25[a]	29.7%	233.7 ± 44.7	1.93 ± 0.22[a]	79.0%	0.77 ± 0.06[a]	54.3%
Omeprazole	10	2.54 ± 0.4[b]	51.1%	1.97 ± 0.14[b]	27.8%	158.5 ± 22.79[a]	1.87 ± 0.13[b]	86.3%	0.72 ± 0.05[b]	65.2%

These data were obtained from groups of 10 rats. Values are represented as mean ± SE. [a]$P < 0.05$, [b]$P < 0.01$ *vs* the positive control (Mann–Whitney *U* test). I: Inhibition; ELI: Esophageal lesions index; MDA: Malondialdehyde; SH: Sulfhydryl.

group (Table 1).

Effects on oxidative markers

Experimentally induced GER increased significantly the MDA and SH concentrations in the esophageal homogenates of the positive control as compared to negative control group; a change also ameliorated by D-002 (25, 100 and 200 mg/kg) and omeprazole (10 mg/kg). Oral treatment with D-002 (25, 100 and 200 mg/kg) decreased significantly the esophageal levels of MDA (64%, 72.5% and 79%, respectively) and SH (32.6%, 50% and 54%, respectively). No dose-effect relationship, however, was seen. Oral omeprazole (10 mg/kg) reduced significantly MDA (86.3% decrease) and SH (65.2% decrease) levels in the esophageal tissues of rats with experimentally induced GER (Table 1).

DISCUSSION

The results of this study demonstrated, for the first time, that oral supplementation of D-002 significantly ameliorated GER-induced esophageal damage in rats. Our data confirm that GER induction causes esophageal lesions and increases the volume of gastric secretion, gastric acidity, and the extent of lipid peroxidation and protein oxidation in esophageal homogenates, as demonstrated by the increased levels of MDA and SH groups. In addition, omeprazole significantly reduced GER-induced changes, as expected[25], all of which confirms the validity of this model in our experimental conditions.

Oral treatment with D-002 (25-200 mg/kg) significantly (approximately 45%) reduced ELI. A dose of 100 mg/kg achieved the ceiling effect (44.2% decrease) and 200 mg/kg produced roughly the same effect (46.1% decrease), which in turn was similar to the reduction (51.1%) induced by 10 mg/kg omeprazole.

All doses of D-002 failed to modify the acidity of the gastric secretion, in agreement with previous results, in the model of pylorus-ligation-induced ulcers in rats, confirming the cytoprotective effect of D-002[20,21]. The highest dose (200 mg/kg) reduced significantly (29.7%) the gastric secretion volume, as did omeprazole (27.8%), but by different mechanisms as proven by the marked reduction of the acidity produced by this drug. The mechanism by which D-002 produces this effect has not been explained to date.

GER-induced ELI has been shown to correlate with the increased production of free radicals that triggers membrane lipid peroxidation, impairs cell functions, and reinforces the oxidative stress through the production of lipid-derived radicals[31]. Accordingly, natural approaches with antioxidant effects have been investigated for the management of GERD[32], and the efficacy of PPIs on mucosal protection is currently explained by the inhibition of acid secretion, as well as its antioxidant and anti-apoptotic effects[33].

Our results support that D-002 was able to attenuate the GER-induced increase in MDA and SH concentrations to 72.5% and 50%, respectively. A dose of 100 mg/kg was the maximal effective dose for both effects, and its effects on oxidative markers were more pronounced than its ability to lower ELI. D-002 was effective at reducing GER-induced esophagitis and the increased oxidative stress in this model, without modifying gastric secretion acidity.

Although the safety of current first-line therapies for GERD is good, the lack of antisecretory activity of D-002 could be beneficial for avoiding the adverse effects associated with long-term acid suppression[15-18,35]. Experimental toxicology has demonstrated the safety of acute, subchronic and chronic oral administration of D-002. A dose of 1000 mg/kg was found to have no observable adverse effects in a long-term (1 year) study in rats[36], and the same was true for a dose of 250 mg/kg in beagle dogs[37]. Clinical studies have shown that D-002 is safe and well tolerated when administered in the short and long term to humans[38-42].

Nevertheless, the present results are a preliminary demonstration of the efficacy of oral D-002 treatment in an experimental model of GER. The efficacy and safety of D-002 for GERD management, therefore, merits extensive further experimental and clinical research.

In conclusion this study demonstrates that acute oral administration of D-002 (25-200 mg/kg) was effective to reduce esophageal lesions and oxidative stress markers in rats with experimentally induced GER, without modifying gastric secretion acidity.

COMMENTS

Background

Beeswax alcohols (D-002), a food supplement, has been shown to produce gastroprotective effects in experimental and clinical studies, but its efficacy against gastroesophageal reflux (GER) has not been investigated before. The authors investigated the effects of a single oral administration of D-002 in a model of experimentally induced GER in rats.

Research frontiers

Several experiments have reported that D-002 protects against gastric mucosa ulceration induced by different agents through multiple mechanisms, including an increase in gastric mucus secretion, improvement of mucus quality, and antioxidant effects on the gastric mucosa. It is unresolved whether D-002 can protect against GER.

Innovations and breakthroughs

This is believed to be the first study to show that acute oral administration of D-002 reduced esophageal lesions as well as esophageal concentrations of malondialdehyde and sulfhydryl groups in rats with experimentally induced GER, without modifying gastric secretion acidity. These findings suggest that supplementation with D-002 protects against esophageal injury induced by GER in rats.

Applications

D-002 is a dietary supplement that has gastroprotective and antioxidant effects. However, its potential effect on GER, a common condition in routine practice, has not been investigated before. This study is a preliminary step in demonstrating whether D-002 could be an alternative to help manage GER. However, extensive experimental and clinical research is still required to demonstrate its application in this field.

Terminology

The esophageal lesion index is a validated score for quantifying the severity of the macroscopic lesions present in the esophageal tissue.

Peer review

This study evaluated the effect of D-002, a defined mixture of higher primary alcohols purified from bees wax, on experimentally induced GER in rats. The possible influence and protective effect of D-002 on experimental gastric ulcer and colitis in rats and on nonalcoholic fatty liver disease in humans has been evaluated by the same group of researchers in recent years. The anti-inflammatory activity of D-002, by reducing the generation of harmful hydroxyl radicals, has been well studied, but it is difficult to acknowledge this substance as a further option in the management of GERD.

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