D-002 treatment attenuates esophagitis in a model of chronic gastro-esophageal reflux in rats.

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Abstract: To investigate the effects of D-002 (beeswax alcohols) on esophagitis induced by chronic gastroesophageal reflux (c-GER) in rats. Rats were randomized into a sham and five groups subjected to c-GER: a positive vehicle control, three D-002 (50, 100 and 200 mg/kg), and one omeprazole (20 mg/kg) group, all treated orally for seven days. c-GER was induced by ligation of the junction between the forestomach and the duodenal side of the pylorus. Esophageal lesions index (ELI), esophageal malondialdehyde (MDA) and sulphydryl groups (SHG) concentrations were assessed. The positive control group exhibited macroscopically signs of esophageal injury assessed in term of ELI, which was significantly higher than in the negative control. D-002 (50, 100 and 200 mg/kg) reduced the ELI showing 30.5, 72.9 and 76.4% protection, respectively; and also significantly attenuated the increased MDA (37.4, 63.6 and 94.2%, respectively) and SHG (16.6, 41.6 and 72.9%, respectively) esophageal concentrations versus the positive control. Omeprazole decreased the ELI (80.2%), MDA (99.3%) and SH (85.4%) esophageal concentrations. As conclusions, this study suggest that repeated oral administration with D-002 protects against reflux esophagitis and decreases esophageal lipid peroxidation and protein oxidation markers in rats with c-GER.

Key words: D-002, beeswax alcohols, esophagitis, chronic gastroesophageal reflux (c-GER), oxidative stress, rats.

I. Introduction

Gastroesophageal reflux disease (GERD) is a common chronic and relapsing disease that results when the gastric acid flows back into the esophagus [1-3]. The incidence and complications of GERD have been dramatically increasing worldwide, being estimated that GERD affects up to 25% of the western population [4]. This situation is of relevant concern due to the link of GERD with the development of Barrett esophagus and the subsequent increased risk of esophageal cancer [5,6].

Despite the etiology of GERD is complex and heterogeneous, it is known that it comes from weak antireflux barriers at the gastro-esophageal junction that become insufficient to protect against increased reflux, thus leading to esophageal damage; reinforced by the unbalance between aggressive (refluxed gastric acid secretion, duodenal juice) and defensive factors (esophageal acid clearance, esophageal tissue resistance) [2,7,8]. Also, GERD is among common gastrointestinal diseases that share inflammation as a pivotal trigger for their development and that display increased oxidative stress as a general outcome. In turn, the reflux-induced increase of inflammatory mediators and reactive oxygen species contribute to the esophageal damage [9-12].

The first step in the management of GERD is lifestyle modification, focused in elevation of the head of the bed, weight loss in obese patients and to avoid foods that may trigger GERD symptoms [13]. Lifestyle measures alone cannot control GERD symptoms and complications, so that pharmacological intervention aimed at reducing gastric acidity with proton pump inhibitors (PPI) or H\(_2\) receptor antagonists (H\(_2\)-RA) is the cornerstone of GERD therapy [14 – 18].

Besides, despite the proven efficacy of such treatments, which can reduce periods of active disease, symptoms and damage persist and recur in many patients [14 – 18]. Also, although PPI and H\(_2\)-RA exhibit a good safety profile, recent data suggest a link between PPI use and some long-term adverse side effects of relevance, like the increased risk of fractures and of the susceptibility to some infections, mainly in the elderly [18 – 21]. The benefits of current therapy to manage GERD then overcome the risks, but the search of new effective and safer treatments is updated. D-002, a mixture of six high molecular weight primary alcohols purified from the beeswax [22], has been shown to exert gastroprotective effects through multiple mechanisms [23 – 27], but without depressing acid secretion [23, 24]. These mechanisms involve the improved quality (content of proteins, glycoproteins and sulfated macromolecules) and increased secretion of the gastric mucus [23 – 25] and anti-inflammatory and antioxidant effects on the gastric mucosa [26, 27]. Indeed, D-002 reduced
hydroxyl radicals [24], malondialdehyde (MDA) (a lipid peroxidation marker) [26, 27] and carbonyl groups (a protein oxidation marker) concentrations and mieloperoxidase (MPO) activity (a marker of inflammation) in vivo, while increased the activity of antioxidant enzymes (glutathione peroxidise –GSHPx-, superoxide dismutase –SOD- and catalase –CAT-) in the gastric mucosa of rats with indomethacine-induced ulcers [27]. In addition, a recent study demonstrated that acute oral treatment with D-002 reduced esophageal lesions and the increase of oxidative stress induced by acute gastric esophageal reflux (GER) in rats, without modifying gastric secretion acidity [28]. The effects of D-002 on chronic GER (c-GER), however, had not been explored. This study was then undertaken to investigate whether D-002 could ameliorate reflux esophagitis in experimentally induced c-GER in rats.

II. Materials and Methods.

Male Sprague Dawley rats (270-300 g) acquired in the National Centre for Laboratory Animals Production (CENPALAB, Havana, Cuba) were adapted for 7 days to the experimental conditions: temperature 25 ± 2 °C, humidity 60 ± 5% and light/dark cycles of 12 h. Food (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 according to the Cuban Guidelines of Animals Handling and the Cuban Code of Good Laboratory Practices (GLP), 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.

2.1, Chemicals and test substance

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 tetratriacontanol (2.6%). Purity (total content of these alcohols) was 90.0%. Omeprazole (OMP), the reference substance, was purchased from DOMER (Mexico).

2.2, Dosage and administration

Both D-002 and OMP were suspended in 1% acacia gum/water vehicle. Rats were randomized into six groups of 10 rats each: a negative vehicle control and six subjected to c-GER induction: a positive vehicle control, three treated with D-002 (50, 100 and 200 mg/kg, respectively), one with OMP 20 mg/kg. Treatments (D-002, vehicle, OMP, were intragastrically administered (1 mL/200 g of bodyweight) once daily for seven days, starting one hour later the surgical induction of cGER. The doses of D-002 are within the range of effective doses in the model of acute GER [28], meanwhile the dose of OMP is that reported as effective in a model of GER in rats [29].

2.3, Induction of chronic gastro-esophageal reflux (cGER)

The induction of c-GER in rats was surgically induced according to Asaoka et al (2009) [30]. In brief, rats fasted for 24 h and then were anesthetized with thiopental anaesthesia (30 mg/kg i.p.), when a midline incision was performed. The stomach and duodenum were exposed extracorporeally, and a transitional (boundary) section from the forestomach to the glandular stomach was ligated using 2–0 silk thread. The duodenal side of the pylorus was covered with a 2-mm-wide 18-Fr nelaton catheter. The stomach and duodenum were returned into the abdominal cavity, which was then closed. After surgery, rats were fasted for a further 24 h (resulting in a total fasting of 48 h). After seven days on treatments, rats were euthanized, the esophagus were removed, incised lengthwise and then the macroscopic esophageal lesions were observed under a magnifying glass and measured. The esophageal tissue was stored at -20 °C until performing the biochemical analyses.

2.4, Esophageal Lesions index (ELI)

The esophageal lesions index (ELI) score was calculated (macroscopic degree of injury 0-6) after gross inspection of the esophagus under a magnifying glass (3 x) by two independent blinded observers. The lesions were graded with a five scores scale: 0: no visible lesions; 1: some erosions and bleeding; 2: total area of lesions < 15 mm²; 3: total area of lesions < 30 mm²; 4: total area of lesions < 40 mm²; 5: total area of lesions < 45 mm²; 6: perforation [31].

2.5, 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 (1g of tissue/9 mL of buffer) by Ultra-Turrax homogenizer T25 (Germany). The homogenates were centrifuged at 5000 x g for 10 min at 4 °C, and the supernatants stored at -80 °C to the analyses. All the assays were conducted by triplicate in an Ultrospec Plus LKB spectrophotometer (Pharmacia LKB Biotechnology, Uppsala, Sweden). Protein concentrations were measured by a modification of the Lowry method Marxwell, 1987 (32).
2.5.1. Lipid peroxidation (LP) assessment: MDA levels in esophageal homogenates were measured as thiobarbituric acid reactive species (TBARS) [33]. In brief: homogenate aliquots (1 mL) were added to a mixture containing 0.2 mL of 8.1% sodium dodecylsulphate (SDS) plus 1.5 mL of 20% acetic acid solution adjusted to pH 3.5, 1.5 mL of thiobarbituric acid (TBA) solution and 1 mmol/L butylated hydroxytoluene, heated at 95 °C for 45 min and cooled. One (1) mL of distilled water plus 5 mL of 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 malondialdehyde bis (dimethyl acetal) as standard. TBARS concentrations were expressed as nmoL of MDA/mg of protein.

2.5.2. Protein oxidation assessment. Esophageal concentrations of sulfhydryl groups (SHG) were measured through the 5′5′-dithio-bis (2-nitrobenzoic acid) (DTNB) assay [34]. Homogenate aliquots (200 μL) were treated with 600 μL of 20 mmol/L Tris-EDTA buffer (pH 8.2), 40 μL of 10 mmol/L DTNB and 3.16 mL of absolute ethanol. This mixture was then incubated at ambient temperature for 20 min and centrifuged at 3000 x g for 10 min. The optical density of the supernatant was measured at 412 nm, using a 13.600 cm coefficient of absoritivity and SH concentrations were reported in mmol/L.

2.6. Statistical analysis

Data were expressed as the mean ± SE. Paired comparisons between control and treated groups were done with the non parametric Mann-Whitney U-test. The level of statistical significance was set at α= 0.05. The analyses were done by using the Statistic software for Windows (Release 4.2, Stat Soft, Inc USA). Dose-effect relationships were assessed by using dose regression linear analysis on the Primer of Biostatistics program (Stanton A, Glantz: copyright (c) 1992, McGraw-Hill, Inc Version 3.01).

III. Results

3.1. Effects on esophageal lesions

Table 1 summarizes the results of this study. Seven days after the surgical procedure, the positive control group exhibited macroscopical lesions that were quantitatively assessed in term of ELI values, which were significantly higher than in the group of negative controls, which did not present visible lesions. Oral treatment with D-002 (100 and 200 mg/kg) for seven days from the surgery day significantly reduced the severity of cGER-induced esophagitis by 72.9 and 76.4%, respectively, as compared to the positive control group, while the lowest dose (50 mg/kg) did not decrease significantly the esophageal injury. The effects of D-002 (50–200 mg/kg) on ELI, however, were not dose-dependent. OMP 20 mg/kg reduced significantly the ELI by 80.2% as compared to the positive control group.

3.2. Effects on oxidative markers

The positive control group subjected to c-GER and treated orally with the vehicle only displayed significant increases in the esophageal concentrations of MDA and SHG as compared to negative control group, changes that were also ameliorated by D-002. Oral treatment with D-002 (50, 100 and 200 mg/kg) decreased significantly, dose-dependently (p<0.05; r=0.968) and markedly (37.4, 63.6, 94.6%, respectively) MDA concentrations. D-002 also reduced significantly SHG concentrations (16.6, 41.6 and 72.9%, respectively), but not in a dose-dependent fashion.

Oral OMP (20 mg/kg) produced significant and marked reductions of MDA (99.3%) and SHG (85.4 %) concentrations in the esophageal tissue of rats with experimental cGER.

IV. Discussion

The results of this study support that oral repeated administration of D-002 ameliorated the esophageal damage induced by c-GER in rats, a fact consistent with the ability of acute D-002 treatment for reducing the reflux esophagitis induced by acute GER in rats [28]. These results expand, therefore, the knowledge on the esophageal protective effect of D-002 to the scenario of chronic reflux, a condition more similar to that found in the clinical practice [2] The ligation of the forestomach and pyloric end induced in this model tend to propagate the inflammatory lesions, which mainly result from the reflux of gastric content [35]. In such regard, the surgical induction of cGER produced macroscopical lesions characteristic of esophageal inflammation, so that ELI values in the positive control group were greater than in the negative control. Also, cGER induction increased the esophageal concentrations of lipid peroxidation and protein oxidation markers (MDA and SHG, respectively), changes that were reduced by omeprazole, the reference treatment, as expected [36]. Such facts confirm the validity of this model in our experimental conditions. Oral doses of D-002 (100 and 200 mg/kg), repeated for seven days, significantly and markedly (≥ 76%) reduced ELI values. Dose-dependence of the effect, however, was not seen, since the lowest dose of D-002 (50 mg/kg) was not effective, and the effects of 200 mg/kg and 100 mg/kg, were statistically similar, as occurred in the study of acute GER in rats [28].
The reduction of ELI by omeprazole (± 80%) was similar to that achieved with D-002, which suggests that the effects of D-002 on reflux esophagitis conditions could be clinically meaningful, a potential benefit that requires extensive clinical investigation.

The present results constitute the first demonstration of the esophageal protective effects of D-002 in conditions of chronic reflux esophagitis in rats. The current data on D-002 (50 – 200 mg/kg) agree with those found in acute GER in rats [28], but the reduction of ELI here seen with 200 mg/kg (± 76%) was greater than that reported in the acute model (± 45%). The fact that in this study we have used repeated instead of single oral doses could have contributed, at least in part, to this difference. Omeprazole also exhibited a greater efficacy in this study than in the previous one, but in this case, in addition of having use repeated dosing, we have used a dose (20 mg/kg) that doubled the previous one (10 mg/kg) [28].

Oxidative stress and increased production of reactive oxygen species have been linked with the development of reflux esophagitis and its potential complications, like Barrett esophagus and esophageal cancer in humans and experimental models [37 – 42]. In agreement with this, marked increases of MDA and SHG esophageal concentrations were induced by c-GER in the positive control group.

Treatment with D-002 (100 and 200 mg/kg) reduced remarkably (to about 95%) the esophageal levels of MDA (a well known marker of lipid peroxidation), and a similar, but less marked reduction was observed Regarding to the concentrations of SHG, which lowered by about 73% with 200 mg/kg. As happened with the effects on ELI, the decreases of the oxidative variables achieved by D-002 were greater than those achieved in the previous study conducted in a model of acute GER, in which MDA and SHG lowered by about 79 and 54%, respectively. The reductions of such markers (about 99% for MDA, 85% for SHG) with omeprazole were apparently, but not significantly, greater than the decreases achieved with the highest dose of D-002. The effects of omeprazole on the esophageal concentrations of MDA and SHG found in this model were greater than those found in the study of acute reflux esophagitis [28]. Then again the difference in the dose scheme should influence, at least partially, the results of the better efficacy of D-002 on c-GER with regards to the acute model, whereas in the case of omeprazole the higher dose here used should be a relevant factor for a better efficacy.

Overall, these results support that the esophageal protective effect of D-002 here demonstrated may be attributable, at least in part, to its ability for lowering oxidative stress markers, in line with the results of the acute study [28]. In such regard, these results are also consistent with the evidences of the efficacy of some antioxidant substances for reducing reflux esophagitis [39, 40].

The fact that D-002 had been able to provide esophageal protection, not only against acute GER, but also in the model of c-GER used in this study is promising and merits further studies. In such regard, the potential contribution of other mechanisms, the use of other experimental models and finally, the proof of concept in clinical practice indicate the long way needed for demonstrating with evidences whether D-002 may play a role in the management of GERD disease.

In conclusion, the present data indicate that oral repeat doses of D-002 protect against experimentally-induced esophagitis and decreases esophageal markers of lipid peroxidation and protein oxidation in rats with experimentally induced c-GER.

REFERENCES

D-002 treatment attenuates esophagitis in a model of....


Table 1. Effects of ELI and esophageal concentrations of oxidative variables in rats with cGER

<table>
<thead>
<tr>
<th>Groups</th>
<th>Doses (mg/kg)</th>
<th>ELI (mean ± SE)</th>
<th>MDA (mmol/mg protein)</th>
<th>SH (mmol)</th>
<th>I (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>-</td>
<td>0.00 ± 0.00</td>
<td>4.39 ± 0.38</td>
<td>0.36 ± 0.44</td>
<td>5</td>
</tr>
<tr>
<td>Positive control</td>
<td>-</td>
<td>3.7 ± 0.69</td>
<td>7.33 ± 0.38</td>
<td>0.84 ± 0.01</td>
<td>10</td>
</tr>
<tr>
<td>D-002 + cGER</td>
<td>50</td>
<td>2.57 ± 0.61</td>
<td>6.23 ± 0.14</td>
<td>0.76 ± 0.03</td>
<td>16.6</td>
</tr>
<tr>
<td>D-002 + cGER</td>
<td>100</td>
<td>1.00 ± 0.43</td>
<td>5.46 ± 0.15</td>
<td>0.64 ± 0.03</td>
<td>41.6</td>
</tr>
<tr>
<td>D-002 + cGER</td>
<td>200</td>
<td>0.87 ± 0.39</td>
<td>4.56 ± 0.17</td>
<td>0.49 ± 0.02</td>
<td>72.9</td>
</tr>
<tr>
<td>OMP + cGER</td>
<td>20</td>
<td>0.73 ± 0.36</td>
<td>4.41 ± 0.10</td>
<td>0.43 ± 0.01</td>
<td>85.4</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SE

cGER: chronic gastroesophageal reflux, ELI: esophageal lesions index, I (%): Inhibition percent, MDA: malondialdehyde, SH: sulphhydryl groups

Data obtained from groups of ten rats each (n=10)

\( ^a P < 0.05; ^b P < 0.01; ^c P < 0.001 \) vs the positive control (Mann Whitney U test)