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Original Research Article

EFFECTS OF THE COMBINED THERAPY D-002 PLUS GRAPE SEED EXTRACT (GSE) ON ASPIRIN INDUCED GASTRIC ULCER IN RATS.

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Abstract

Non-steroidal anti-inflammatory drugs represent a pivotal cause of gastric ulceration. Gastroprotective effects of D-002 and grape seed extract (GSE) monotherapies have been documented, but the effects of their combined therapy remained unexplored. This study investigated the effects of administering together D-002 plus GSE on aspirin-induced gastric ulceration in rats. Two experiments were conducted for investigating the effects of GSE alone (1, 5 or 25 mg/kg) (first experiment) and of the combined therapy D-002 (5 mg/kg) plus GSE (1 mg/kg) (second experiment) on aspirin-induced gastric ulcers in rats. Effects on gastric juice volume and gastric mucus quantity were also determined. GSE (1, 5 and 25 mg/kg) significantly reduced (34%, 68.9% and 73.1%, respectively) ulcer indexes. Low doses of D-002 (5 mg/kg) and GSE (1 mg/kg) decreased significantly but modestly (36.3% and 38%, respectively) ulcer indexes, while same doses given as combined therapy reduced significantly and markedly (90.5%) gastric ulceration versus the control group, and versus each monotherapy. D-002 (5 mg/kg) and the combined therapy increased significantly mucus secretion, but all treatments failed to modify gastric juice volume. Concluding, the combined therapy D-002 plus GSE inhibited aspirin-induced gastric ulceration in rats more effectively than each monotherapy, the interaction being of synergic nature.

Key words: D-002, grape seed extract, combined therapy, gastric ulcer.

Introduction

Peptic ulcer is a common gastrointestinal pathology that can occur as gastric or duodenal ulcer. Gastric ulcer, a discontinuity in the gastric mucosa penetrating through the muscularis mucosa,[1] results from the imbalance between aggressive (acid, pepsin, *Helicobacter pylori*, nonsteroidal anti-inflammatory drugs –NSAIDs–, ethanol) and defensive (bicarbonate, mucus secretion, blood flow, cellular regeneration, endogenous protective agents like epidermal growth factors and prostaglandins –PG–) factors acting on the gastric mucosa.[2-5] Despite remarkable advances in their management, peptic ulcers can lead to sometimes life-threatening complications.[6] NSAIDs, commonly prescribed to manage pain

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and inflammatory conditions, [7] represent a pivotal cause of gastric ulceration, mainly in the elderly. [8,9] Although NSAIDs are very effective for treating inflammation, their use is associated with adverse effects that limit that range from dyspepsia to ulceration and complications, such as hemorrhage or perforations. [10, 11]

The pathogenesis of NSAIDs-induced gastric ulceration mainly involves the inhibition of cyclooxygenase (COX) activity and then of PG synthesis, which in turn leads to decrease gastric mucus and bicarbonate secretion, mucosal microcirculation, to increase acid and pepsin secretion, and to impair microvascular structures. In addition, NSAIDs-induced gastric ulceration includes the increase in the production of reactive oxygen species (ROS), lipid peroxidation and neutrophil infiltration. [12-16] Bearing in mind these facts, the search for new strategies that ameliorate NSAIDs-induced gastric damaged is updated.

D-002, a mixture of six high molecular weight fatty alcohols purified from the beeswax, has been shown to produce anti-inflammatory, antioxidant and gastroprotective effects in experimental and clinical studies. [17-25] In particular, the gastroprotective effects of D-002 comprise a multiple mechanism that involves the increased secretion and improved quality of the gastric mucus, [20, 21] as well as antioxidant effects and reduction of neutrophil infiltration in the gastric mucosa, demonstrated in rats with NSAIDs-induced acute gastric ulceration. [26-28]

Grape seed extract (GSE), a mixture of polyphenols isolated from the grape (*Vitis vinifera*) seed, approved as antioxidant supplement in some countries,[29,30] exhibits PG-dependent gastroprotective effects, associated also to the decrease of lipid peroxidation.[31, 32]

D-002 and GSE have proven to display similar antioxidant effects, [33] and the antioxidant efficacy of their combined therapy is superior to that of the respective monotherapies. [34] The gastroprotective effects of such combined therapy, however, had not been studied yet.

In light of these issues, this study was undertaken to investigate the effects of combined therapy of D-002 and GSE on aspirin induced gastric ulcer in rats.

Materials and Methods

Animals

Male Sprague Dawley rats (250-300g) were purchased from the National Center for Laboratory Animal Production (CENPALAB, Havana, Cuba) and adapted for 7 days to the following conditions: temperature (22-23 ^{o}C), relative humidity (55-60%) and 12 hours dark/light cycles. Food and water were freely supplied. The animals fasted for 24 hours prior to the experiments.

The experiments were performed after obtaining the approval of the Institutional Board for animal use, and were conducted to the Cuban Guidelines for the Care of Laboratory Animals and the Cuban Code of Good Laboratory Practice (GLP).

Administration and dosage

The batch of D-002 (030040109), supplied by the Plants of Natural Products (National Center for Scientific Research, Havana, Cuba) was used after corroborating its quality criteria. Batch composition, assessed with a validated gas chromatographic method, [35] was as follows: 1-tetracosanol (5 %), 1-hexacosanol (10.2 %), 1-octacosanol (14 %), 1-triacontanol (34.21 %), 1-dotriacontanol (24.24 %) and 1-tetratriacontanol (3.03 %). Purity (total content of these alcohols) was 90.7%. GSE (batch: R3992407), 85% in proanthocyanidine content (Blackmores, Sydney, Australia) was used in the experiments.

Both D-002 and GSE were suspended in 1% Arabic gum/water.

Two experiments were performed. A first experiment evaluated the effects of different doses of GSE on aspirin-induced gastric ulcers in order to determine the submaximal doses to be used in the second experiment, which in turn evaluated the effects of the combined therapy D-002 plus GSE on aspirin induced gastric ulcer in rats.

Effects of GSE on aspirin-induced gastric ulcers

Rats were randomized into four groups of 10 rats each: a control group treated with the

vehicle and three with GSE (1, 5 or 25 mg/kg, respectively).

All treatments (vehicle, D-002 and GSE) were administered as single doses by gastric gavage (5mL/kg) one hour before oral administration with aspirin (300 mg/kg). [36] The animals were sacrificed after five hours under an overdose of thiopental anaesthesia. Their stomachs were washed with cold saline and removed for examining the ulcer index macroscopically.

Effects of combined therapy of D-002 and GSE on aspirin-induced gastric ulcers

Rats were distributed randomly into four groups that received orally aspirin (300 mg/kg) for gastric ulcer induction: a vehicle control and three groups treated with D-002 (5 mg/kg), GSE (1 mg/kg) and the combined therapy of D-002 (5 mg/kg) + GSE (1 mg/kg), respectively.

The dose of 5 mg/kg of D-002 was selected from previous dose-ranking in house studies wherein it produced a modest reduction of aspirin-induced ulcers in rats, since higher doses produced a marked inhibition.[28, 37, 38]

In this experiment, effects on gastric juice volume and gastric mucus secretion were also determined.

Evaluation of gastric ulceration

The lesions in the gastric mucosa were examined macroscopically using magnification 3x. Ulcer indexes were determined by the sum of the lengths of the whole gastric lesions (in mm). Two independent, blinded observers performed the observations and measurements of lesion lengths.[39]

Gastric juice volume quantification

After stomachs were removed the gastric juice content was extracted, centrifuged at 1000 rpm during 10 min, and the volume was measured in the graduated cylinder tube.

Gastric mucus quantification

The stomachs were weighed, opened by the higher curvature and then the mucus was gently scraped with a scalp. Immediately, the mucus content was weighed and the relation mucus weight (mg)/stomach weight (g) (MW/SW) was expressed.

Statistical analyses

Comparisons among groups were done with the Kruskal Wallis test; while the Mann-Whitney U test was used for paired comparisons between each treated and control groups. Statistical significance was chosen for $\alpha = 0.05$. Data were processed with the Statistics Software for Windows (Release 6.1 Stat Soft Inc, Tulsa OK, USA).

Results

Table 1 shows the effects of GSE on aspirin-induced gastric ulcer in rats. Oral acute administration of GSE (1, 5 and 25 mg/kg) significantly and markedly reduced the ulcer indexes by 34%, 68.9% and 73.1%, respectively, but not in a significant dose-dependent manner.

Table 2 shows the effects of combined therapy. The administration of D-002 (5 mg/kg) and GSE (1 mg/kg) alone significantly inhibited the gastric ulcer indexes (36.3% and 38%, respectively). The combined therapy with D-002 plus GSE produced a marked (90.5%) inhibition statistically significant as compared to the control group and to each monotherapy. As can be seen, D-002 (5 mg/kg) monotherapy and the combined therapy significantly increased the mucus quantity as compared to the control group, but their effects on this target were statistically similar. None of the treatments affected the gastric juice volume.

Discussion

The present study demonstrated that the oral acute administration of D-002 plus GSE significantly and markedly prevented against gastric ulceration induced with aspirin in rats, being superior in efficacy as compared to each monotherapy.

Independent administration of both monotherapies accounted for modest reductions of ulcer indexes of 36.3% and 38 % with D-002 (5 mg/kg) and GSE (1 mg/kg), respectively. By contrast, the decrease of aspirin-induced gastric ulceration with the combined therapy was marked (90.5%). This result indicates a synergic interaction between both substances, since the antiulcer efficacy of

the combined therapy was superior to the sum of the effects of both mono-therapies (74%).

As expected, oral treatment with aspirin (300 mg/kg) induced characteristic gastric ulcers useful for evaluating potential gastroprotective treatments, [40] which supports the validity of this model in our conditions, and then the results here described.

As a glance, the present results suggest that D-002 and GSE protect against aspirin-induced gastric ulceration through different mechanisms. The effects on the other variables assessed, however, do not explain the nature of the mechanisms that support the observed synergic effect. None of the treatments (D-002, GSE, combined therapy) modified the gastric juice volume, what indicates that their gastro-protective effects do not involve the suppression of the gastric secretion. Moreover, although D-002 and the combined therapy, but not GSE monotherapy, increased the gastric mucus production, the effects of D-002 and the combined therapy were similar, so that this effect doesn't explain the synergism seen with the combined therapy. Then, the reason whereby the interaction between D-002 and GSE on aspirin-induced ulcers in rats is synergic cannot be attributed to anti-secretory or mucus enhancing effects, and remains to be elucidated.

The gastroprotective effect of D-002 has shown to depend of multiple, rather than of single mechanisms. These mechanisms involves the improvement in the quality and the increase of gastric mucus production, [20, 21] and antioxidant effects that includes the inhibition of the production of hydroxyl radicals (OH*) *in vivo*, and the consequent decrease of lipid peroxidation and protein oxidation processes, parallel to the increase on the activity of catalase, glutathione peroxidase and dismutase superoxide enzymes, all demonstrated in the rat gastric mucosa. [27] On its side, antioxidant effects of GSE on the gastric mucosa have been demonstrated. [41]

It should be noted, however, that we previously demonstrated that combined therapy with D-002 and GSE caused antioxidant effects superior to each monotherapy, so that this interaction cannot be discarded as one of the plausible explanation of the synergism here observed. Unfortunately, we did not explore the effects on any oxidative marker, a fact that we acknowledge as a limitation of this study.

Indeed, consequent with its antioxidant effects, the reduction of neutrophil infiltration induced by D-002 in rats with aspirin-induced ulcers is another relevant mechanism that supports its gastroprotective action. [28] In such regard, inflammation and neutrophil infiltration are key factors in the pathogenesis of NSAIDs-induced gastric damage. The inflammation induced in the gastric mucosa by aspirin is accompanied by increased TNF-α production, which augments neutrophil-derived superoxide generation and stimulates IL-1β production, leading to neutrophil accumulation. [42] A reduction of neutrophil infiltration has been associated to the effects of GSE on different targets. [43,44]

The inhibition of neutrophil accumulation in the gastric mucosa of rats with aspirin-induced ulcers is coherent with the dual inhibition of COX and 5-lipooxygenase (5-LOX) induced by D-002.[45] NSAIDs-induced inhibition of COX activity curtails PGs production, and then triggers the metabolism of arachidonic acid towards the overproduction of leukotrienes (LTs), mainly of LTB4, gastrotoxic mediators that increase ROS generation, thus enhancing the gastric damage induced by PGs-deficit. [46, 47] So, the inhibition of LOX produced by D-002 cancels this compensatory switch, thus contributing to decrease NSAIDs- induced gastric damage. Since GSE and red wine polyphenol extracts have been shown to inhibit 5-LOX activity, [48] this effect cannot be argued to enable the synergic effect of D-002 and GSE for protecting against NSAIDs- (particularly aspirin-) induced gastric ulceration.

In light of these grounds, further studies should investigate the roof of such synergism, including not only the search of oxidative markers and neutrophil infiltration in single experiments, but the effects on TNF-α and IL-1β production in the gastric mucosa.

In conclusion, oral administration of the combined therapy D-002 plus GSE protected against aspirin-induced gastric ulceration in rats more effectively than each monotherapy, the interaction showing to be of synergic nature.

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Table 1. Effects of GSE on aspirin induced gastric ulcer in rats.

. ***Groups***	***Doses (mg/kg)***	***Ulcer Index (mm)***	***I (%)***
Control	__	*31.42 ±6.07*	__
GSE	*1*	*20.71 ±3.75**	*34*
GSE	*5*	*9.76 ±1.71***	*68.9*
GSE	*25*	*8.44 ±2.09***	*73.1*

I (%): inhibition percentage,

*** p <0,01; Comparison vs Control*

(Mann Whitney U test)

Table 2. Effects of combined therapy of D-002 and GSE on aspirin induced gastric ulcer in rats.

. **Groups**	**Doses (mg/kg)**	**Index Ulcer (mm)**	**I (%)**	**GJV (mL)**	**I (%)**	MW/SW **(mg/g)**
Control	__	25.06 ± 3.78	--	1.54 ± 0.14	--	92.03 ± 5.13
D-002	5	15.96 ± 1.08*	36.3	1.20 ± 0.20	22.0	119.88 ± 8.67*
GSE	1	15.52 ± 2.32*	38.0	1.48 ± 0.24	3.9	107.61 ± 10.28
D-002+GSE	5 + 1	2.38 ± 0.80 ***ab	90.5	1.37 ± 0.10	11.0	119.19 ± 6.32*

I (%): inhibition percentage, GJV: gastric juice volumen,

MW/SW: Mucus weight/Stomach weigh

*** p <0,01; *** p<0,001 Comparison vs Control, a p<0,001 Comparison vs D-002, b p<0,001 Comparison vs GSE*

(Mann Whitney U test)