ANSWERING REVIEWERS

August 27, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 4567-review.doc).

Title: Protective effects of D-002, on experimentally-induced gastroesophageal reflux in rats.

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

Name of Journal: *World Journal of Gastroenterology*

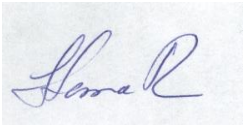
ESPS Manuscript NO: 4567

Dear Reviewer **(**00061678**):**

1) I appreciate very much your comments about our paper.
2) The spelling mistakes were revised and corrected.

Thank you again for evaluating our manuscript in order to be considered for publication in the *World Journal of Gastroenterology.*

Sincerely yours,



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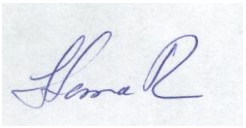
The manuscript has been improved according to the suggestions of reviewers: Editor
1 The paper format has been updated

2 Revision has been made according to the suggestions of the reviewer
(1) We reworded the abstract to comply journal requirements by section
(2) The core TIP was added.
(3) The headings 1 and 2 were corrected in all the text.
(4) Comments, but not peer-reviews, were added too, since we understand what these last ones should be done by the reviewers and/or the editors. Is this correct, please?
(5) References and typesetting were corrected

Thank you for your suggestions for improving our manuscript submitted to the *World Journal of Gastroenterology*.

Sincerely yours,



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Name of Journal: *World Journal of Gastroenterology*

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Dear Reviewer **(**00039422**):**

1) We appreciate your comments about our paper since them have contributed to improve its quality.

2) About your comment "*…it is difficult to acknowledge this substance as a further option in the management of GERD. Any treatment for GERD should be addressed to reduce the possibility of reflux or to protect the mucosa from the refluxate, more than to prevent the inflammation mechanisms consequent to the esophageal injury. In my opinion this is a significant concern. With the available therapeutic options, it is difficult to find a role for D-002, as well as of others anti-inflammatory substances (see ref 30 cited by the authors) and more studies are required before these data can be extrapolated to the recommendation for the use of D002 as a helpful tool for the management of GERD"*

I agree with you about the criterion of "any treatment for GERD should be addressed to reduce the possibility of reflux or to protect the mucosa from the refluxate, more than to prevent the inflammation mechanisms". Keeping in mind such alternative, the case of D-002 mainly falls into the category of preventing the mucosa from the refluxate injury, since previous studies have demonstrated that D-002 has a gastroprotective effect that involves increased gastric mucus secretion (one of the main defensive factors against the acid secretion acting on the gastric mucosa) and improved mucus composition (increased content of mucus proteins, glycoproteins and sulfated macromolecules). In addition, D-002 also exhibits antioxidant and anti-inflammatory effects, and considering that oxidative stress and inflammation are etiological factors involved in the pathogenesis of GERD, such effects could contribute additionally to the gastro-esophageal protection. We believe that our explanation in the introduction was not enough to understand this rationale, so that we have reworded this matter in the new version of the Introduction.

On the other hand, you are right that "more studies are required before these data can be extrapolated to the recommendation for the use of D002 as a helpful tool for the management of GERD". We wrote that sentence to suggest the possibility that D-002 could be useful for treating GERD, but never thinking this was an immediate fact. Then, we deleted such sentence from our conclusions and we restrict our conclusions to the evidences obtained in this work.

3) About your comment *"Considering the chronicity of the disease, a long-term administration of D-002 should be suggested and to my knowledge there is no evaluation in the literature of its possible long- or at least medium term side effects, even experimentally. The paper is describing an acute experiment and it is problematic to transfer the results suggesting that the drug can be useful for the management of a chronic disease".*

We agree that treatments used for managing chronic diseases, as GERD, require the demonstration of their long-term safety. This paper, as the first one demonstrating the efficacy of D-002 in a GERD model, needed to demonstrate its efficacy when administering acutely and did not include the assessment of repeat dosing. Nevertheless, experimental and clinical data support the safety of D-002.

Experimental evidences of the long-term safety of D-002

A set of experimental toxicology studies have demonstrated the safety of oral administration of D-002. A single oral dose of 5000 mg/kg did not produce deaths or symptoms in rats, mice and rabbits. Also, the 90 days study of oral toxicity in Sprague-Dawley rats found that the highest dose tested (625 mg/kg) was a non observable dose effect level (NOAEL), and the same was true for a dose of 1000 mg/kg in the long-term (1 year) study in rats since no treatment-related toxicity was seen, including assessments of the effects on body weight, food consumption, clinical observations, blood parameters, organ to weight ratios and histopathological findings. These results are included in the following paper, referenced in Entrez PubMed:

Rodeiro I, Alemán C, Noa M, Menéndez R, Mas R, Hernández C, Garcia M. "Preclinical oral toxicology in rats of D-002, a natural drug with antiulcer effects". Drug Chem Toxicol. 1998; 21(2):151-62.

In addition, a long-term study (1 year) investigated the oral toxicity of D-002 in beagle dogs, in which the highest dose tested (250 mg/kg) was a NOAEL. So, D-002 was well tolerated throughout the study. There were no deaths and no signs or toxic symptoms were observed. D-002 unaffected weight gain and food consumption, and no hematological, blood biochemical or histopathological disturbances attributable to treatment were observed. Then, this study demonstrated no treatment-related toxicity induced by long-term administration D-002 to beagle dogs, being also available from Entrez PubMed, as follows:

Alemán C, Rodeiro I, Noa M, Menéndez R, Gamez R, Hernandez C, Mas R. One-year dog toxicity study of D-002, a mixture of aliphatic alcohols. J Appl Toxicol. 2001; 21(3):179-84.

Clinical evidences of the D-002 safety in humans

Short-term studies

There are various clinical studies that support the short-term safety of D-002 administered to 12 weeks, summarized below. Some of these papers appear in Entrez PubMed, meanwhile others appear in other journals, including some with recognized impact factor.

- Menéndez R, Mas R, Illnait J, Pérez J, Amor AM, Fernández JC, González RM. Effects of D-002 on lipid peroxidation in older subjects. J Med Food 2001, 4 (2):71-77
- Menéndez R, Mas R, Amor AM, Perez Y, González RM, Fernández JC, Jiménez S. Antioxidant effect of D-002 on the in vitro susceptibility of whole plasma in healthy volunteers. Arch. Med Res. 2001, 32:436-441.
- López E, Illnait J, Molina V, Oyárzabal A, Fernández L, Pérez Y, Mas R., Mesa M, Fernández J, Mendoza S, Gómez M, Jiménez S, Ruiz D. "Effects of D-002 (beeswax alcohols) on lipid peroxidation in middle-aged and older subjects" Lat Am J Pharm 2008, 27: 695-703.
- Rodríguez I, Illnait J, Molina V, Oyarzabal A, Fernández L, Fernández J, Mesa M, Mas R, Mendoza S, Gámez R, Jimenez S, Ruiz D. "Comparison of the antioxidant effects of D-002 (beeswax alcohols) and grape seed extract (GSE) on plasma oxidative variables in healthy subjects" Lat Am J Pharm 2010, 29: 255-262.

Medium (24 weeks) and long-term studies

There are three clinical studies that support the medium (24 weeks) and long-term (3 years) safety of D-002, summarized below. One of these papers appear in Entrez PubMed, other appear in a journal with recognized impact factor and the third one in a local journal.

- Illnait J, Rodríguez I, Mendoza S, Fernández Y, Mas R, Miranda M, Piñera J, Fernández JC, Mesa M, Fernández L, Carbajal D, Gámez R. Effects of D-002, a mixture of high molecular weight beeswax alcohols, on patients with non-alcoholic fatty liver disease (NALFD). KJIM 2013; 28(4):439-448
- Illnait J, Rodríguez I, Molina V, Mendoza S, Mas R, Fernández L, Oyarzábal A, Pérez Y, Mesa M, Fernández JC, Gámez R, Jimenez S, Ruiz D, Cruz Y. Effects of D-002 (beeswax alcohols) on gastrointestinal symptoms and oxidative markers in middle-aged and older subjects. Lat Am J Pharm 2013, 32: 166-174
- Fernández L, Terry H, Quiñones AM, et al. Effects of Abexol in middle-aged and older subjects: an open follow-up. Rev CENIC Cien Biol 2008, 39: 3 – 8

Note: Lat Am J Pharm is Latin American Journal of Pharmacy (previous Acta Farmaceutica Bonaerense).

Nevertheless, although there are experimental and clinical evidences about the safety of oral intake of D-002, we agree that "it is problematic to transfer the results suggesting that the drug can be useful for the management of a chronic disease". This problem has been solved already by the change in our conclusions, as was referred in the first answer.

4) About your comment "the dosage of omeprazole seems quite elevated, considering that it could account for a 700 mg daily in a 70 kgs adult men. Although this is an acute experiment, the authors should better explain why this dose was chosen".

This dose of omeprazole (10 mg/kg) was chosen in accordance to that reported as effective in rats with experimentally induced GER (Inatomi N et al, Japan J. Pharmacol 1991, 55: 437-451). Rationally, we expected that this dose was also effective in our experimental conditions.

It is important to remark that animal doses should not be merely extrapolated to human doses, taking into account that animals, mainly the rodents, are more resistant to drug effects than the human beings, which is due to different interspecies differences including anatomic, physiological and metabolic differences, among others. These facts support that the choice of the effective doses of omeprazole in rats are greater than in humans and that our study protocol should be based in previous experiences of other authors in experimental models in rats near to that used by us.

5) About your comment *"In any case, such a high dosage reduced ELI in only 50% of animals, not substantially different from D-002 and indeed not a remarkable percentage. You would expect a higher rate of protection with both drugs. In ref. 29, cited as a model for GERD in rats, whose technique is actually different from that described in the manuscript (described in ref 30), the dosage of 1 mg/kg/day was able to prevent esophagitis in a very high percentage of animals".*

There is some relevant misunderstanding about the concept of percent inhibition, probably due to our first English grammar construction. The inhibition percentage achieved by omeprazole (10 mg/kg) or D-002 on ELI (nearly 50%) represents that both substances reduced ELI severity to about the half of that observed in the positive control group, which should represent the 100% of the injury. Then, 50% inhibition does not means that ELI has been reduced "in only 50% of animals," as you understood, but a 50% decrease in the severity of ELI as compared to the positive control group. In such regard, this appreciation is reinforced by the statistical test that we used to compare ELI severity scores (a non parametrical test for the analysis of continuous data), meanwhile the comparison of animals with ELI should require the use of Fisher Exact Probability test, which was not done.

As we referred above, an oral dose of omeprazole 10 mg/kg is within the range of effective doses in rats with experimentally induced GER (Inatomi N et al, Japan J. Pharmacol 1991, 55: 437-451) rather than a high dose.

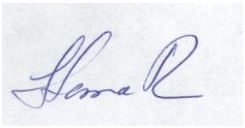
In summary, taking into account: a) the degree of severity of GER-induced ELI in this work (> 5, 6 being the highest score), b) that both D-002 and omeprazole were administered acutely, and c) that the reduction of ELI severity versus the positive control induced by these treatments was almost the half, a protection clinically meaningful may be expected with repeated administration of the treatments, but this assumption (as the opposite) requires evidences including studies with repeated dosing and chronic models of GER.

You are right that the original reference No 29 was wrong for justifying the effective dose of omeprazole because that study used lanzoprazole and found a high inhibition percent with 1 mg/kg/day of lanzoprazole, another proton pump inhibitor, but after being administered for a longer period (3 weeks) not acutely. Our mistake was corrected already, as the right reference is Inatomi et al, 1991 who investigated doses up to 30 mg/kg, **and found that the ID50 value was 13.7 mg/kg, near to the dose selected by us**

6) References and typesetting were corrected

Thank you for your comments in order to improve the quality of our manuscript submitted to *World Journal of Gastroenterology*.

Sincerely yours,



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August 27, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 4569-review.doc).

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

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

Name of Journal: *World Journal of Gastroenterology*
ESPS Manuscript NO: 4569

Dear Reviewer **(**00504637**):**

1) Firstly, thanks for your comments about our paper. They allow improve our work.
2) About your comment "*The background section was too long, should summarized. Authors should avoid report useless literature information in this section".*

In accordance to your suggestion the background section was reduced, reworded, some references were deleted and other new references added in accordance to your queries.

3) About your comment "However the title was accurate, I think it need typing redaction"

The coma between **D-002** and **on** in the original title "Protective effects of D-002 on experimentally-induced gastroesophageal reflux in rats" was eliminated.

4) About your comment *"In abstract 3rd row D003 should change as D002".*

OK, we already replaced D003 by D002 in the abstract, now 5th row.

5) About your comments *"The methodology section was clear, statistical analyses were appropriate. Tables could improve to readability"*

The tables were already corrected according to the format of the journal, which improves the readability.

6) About your comment *"Authors should imply current therapeutic approach and superiority of new therapy".*

The current therapeutic approach had been already briefly summarized in the original Introduction (paragraph 5th) as follows: "Treatment of GERD includes the use of conventional antisecretory treatments aimed primarily at reducing gastric acidity like proton pump inhibitors or H2 receptor antagonists. Though these therapies are effective for a while and help to maintain remission, symptoms are recurrent and patients become refractory and experience drug-related side effects. Then, the search of new effective and safe treatments to manage GERD is a current issue." We reworded the paragraph and added some related references. In fact, the cornerstone of the mechanism of action of these substances is the reduction of the acid secretion, undoubtedly is useful to curtail the reflux as reduce the aggressive action of the gastric acid, but at the same time affect the normal way of the digestion, which requires an acid environment. Then, treatments that reinforce the defensive factors of the mucosa may help the deleterious effect of the refluxate. Keeping in mind these facts and a) that D-002 increases gastric mucus secretion, improves mucus quality and reduces the oxidative stress and inflammation of the mucosa without affect gastric secretion, we expected that it could be an alternative to reduce GERD-related damage, mainly because toxicological and clinical studies have shown that D-002 is safe and well tolerated. The first step in this rationale was to demonstrate whether D-002 could be useful to protect GER in an experimental study, which was the essence of our work, the first study demonstrating this fact.

Nevertheless, although our results were promising since D-002 protected from esophagitis as well as omeprazole 10 mg/kg, this study was not a comparative efficacy study, as we used omeprazole just as reference drug. To make stronger inferences we need conduct first experimental comparative dose-effect studies on GER models and later on randomized and double-blind comparative studies of D-002 vs current therapies for GERD. We have added some of these points within the discussion.

7) About your comment *"Gastro esophageal reflux patients inflammatory mediators, free oxygen radicals, and lipid-protein peroxidation products increase. This increment cannot evaluated as reason of reflux, maybe a results of increased oxidative stress on mucosal damage".*

You are right that the factors mentioned above are not the cause of reflux as they appear as consequence of the mucosal damage. Nevertheless, as a feed back cycle they contribute to the initial mucosal injury. These aspects had been explained, but not clearly, in the original Introduction (paragraph 2th): "Although the etiology of the abnormal reflux of the gastric contents from the stomach to the esophagus is complex and due to multiple causes**, the disease seems to result from weak anti-reflux barriers at the gastro-esophageal junction that become incompetent to protect against increased reflux, thus leading to esophageal erosion and inflammation. The unbalance between aggressive factors (refluxed gastric acid secretion and duodenal juice) and defensive factors (esophageal acid clearance, esophageal tissue resistance) is the reason of esophagus damage."** Nevertheless, we agree that the explanation was not good enough, and we reworded such idea in the new second paragraph, so that we replace the original sentence by ***"On its side, GER-induced increase of inflammatory mediators and reactive oxygen species have been shown to contribute to the mucosal damage".*** Accordingly, for such reasons, we had chosen the ELI score (index of the esophageal mucosal damage produced by the reflux) as the main efficacy variable of the treatment, meanwhile oxidative variables were secondary variables.

8) About your comment "*There is no scientific evidence exist side effect of PPIs except osteoporosis among elder patients. This could be discussed in discussion section."*

PPIs are amongst the most over prescribed drugs in clinical practice and have demonstrated a very good safety profile. Indeed, there is no doubt that the benefits and excellent efficacy of PPIs overcome their risks. Nevertheless, although acid suppression with PPI is the first-line of therapy for reflux disease; despite this, symptoms and injury persist in many patients. On the other side, although the safety of PPIs has been demonstrated not only in clinical studies but in years of experience with millions of users, recent data have shown that PPI use is linked, as you mentioned, with an increased risk of fractures, but also with *Clostridium difficile* infection, community-acquired pneumonia, vitamin and mineral deficiencies, and some drug interactions. It is true, however, that some of these facts remain controversial, but all together open a place for novel treatment approaches, including mono- or/and combined therapies that may help fill this blank, which motivates continue research on this topic. These aspects are included in the new version of both the Introduction and Discussion.

9) About your comment *"Authors should avoid writing any comment in discussion section that not directly related the research."*

In accordance with your suggestion, we have reworded the Discussion, which contains only aspects directly related the research and some required by other reviewer. Some references were deleted and some new were added.

Thank you for your comments in order to improve the quality of our manuscript submitted to *World Journal of Gastroenterology.*

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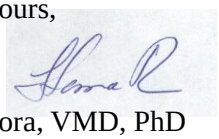

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