

May 14, 2015



Dear Editor,

Please find enclosed the edited manuscript in Word format (answer to reviewers.doc).

**Title:** N-acetylcysteine modulates angiogenesis and vasodilation in stomach such as DNA damage in blood of portal hypertensive rats

**Author:** Francielli Licks, Renata Minuzzo Hartmann, Camila Marques, Elizângela Schemitt, Josieli Raskopf Colares, Mariana do Couto Soares, Juliana Reys, Camila Fisher, Juliana da Silva, Norma Possa Marroni

**Name of Journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO:** 17799

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer (**see below**)

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

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1. *Reviewer 03294162*

The authors conducted a study “N-acetylcysteine modulates angiogenesis, vasodilation, and DNA damage in portal hypertensive rats”. Animals were divided into four experimental groups (n = 6 each): sham-operated (SO), SO + NAC, partial portal vein ligation (PPVL) and PPVL + NAC. NAC (Sigma Chemical Co., St. Louis, MO, USA; CAS registry number 616-91-1) was administrated at a dose of 10 mg/kg, intraperitoneally, dissolved in 0.6 mL of normal saline solution (0.9% NaCl). In my opinion, the article is of great interest and is properly drafted, so I have no doubt that the authors performed an excellent job. The results are quite interesting, showing great effect of NAC in the study. The methodology is clear. The results are displayed clearly, with charts and figures that facilitate the interpretation, however, the graphic pictures are with a bad resolution on the manuscript sent for evaluation. I suggest improvement. I suggest that the introduction is brief, and some paragraphs merged

We have sent the graphic pictures to the editor in other format in order to improve the resolution, as asked by him at the edited-document (powerpoint). Some paragraphs were merged, in order to make the introduction more brief. We deeply appreciate your attention to revise our manuscript.

2. *Reviewer 02861225*

Licks et al conducted a small study on effects of NAC on portal hypertensive gastric mucosa in rats. Since the effects on portal pressure have already been published (Ref #30), the only new results cover eNOS, VEGF and DNA damage. Although results are solid and clear, connections are only made via literature and there are no additional experiments supporting the reported changes/evidence. Please check spelling throughout the manuscript. The abstract is not clearly written, especially the group comparisons which lack units. The abbreviation NTT is not explained. The background is written very broadly and does not cover literature about NAC, VEGF and eNOS sufficiently. The results are written very short. The columns in the figures all have the same pattern and are thus not easily distinguishable between the groups. It is not explained for what group comparison the asterisks stand for. The discussion is written nicely, however the conclusions are somewhat extensive for so this small amount of data. In summary, I would suggest changing the manuscript style to a short report.

In the previous work published by the main author <sup>[1]</sup>, we evaluated the role of portal pressure, oxidative stress and nitric oxide in the experimental model of portal hypertension. Furthermore, the effect of NAC over these parameters. We come to the conclusion that the effects of NAC in this experimental model was due to its effect on the nitric oxide bioavailability by evaluating the nitric oxide metabolites (nitrates and nitrites).

But what would it be the activation pathway of this molecule? Which enzyme is activated in order to increase the NO production in the collateral circulation? What would be the effect of NAC over that pathway?

Those were the questions answered in the present article.

First of all, we couldn't affirm that the animals were portal-hypertensive without measuring of portal pressure on them. So we performed this measured in the animals used in present study in order to confirm the effectiveness of PPVL.

Second of all, the connections made in the present article are not only based on the literature, but also by the results achieved in the study and its relation to the previous one.

It is well-known that nitric oxide (NO) is the main mediator in the vascular abnormalities present in the portal hypertension <sup>[2]</sup>.

In this present study, we demonstrated that eNOS may be responsible for the overproduction of NO. Most important, NAC was able to reduce the eNOS expression, evaluated in this study by *Western blot* and immunohistochemistry.

The overproduction of NO may also be stimulate by the VEGF<sup>[3]</sup>, which presented itself increased in the PPVL group. NAC was also able to decrease this parameter in the PPVL + NAC group. So we believe that the pathway eNOS/ VEGF was modulated by NAC.

The oxidative damage induced by the overproduction of nitric oxide is given by the power of NO to bind with other reactive species of oxygen such as the superoxide anion (O<sub>2</sub><sup>-</sup>) producing highly injurious compounds, such as peroxynitrite (ONOO<sup>-</sup>), leading to nitrosative stress<sup>[4]</sup>.

This reactive specie was evaluated in this study by the NTT levels (nitrotyrosine) by immunohistochemistry. NAC was able to reduce it, reducing therefore oxidative stress. We already added the meaning of the abbreviation in the abstract.

The DNA damage is another way of showing that reactive species of oxygen are being produced in this experimental model, and this damage was also modulated by the antioxidant effects of NAC. DNA damage is one on the markers to evaluate oxidative stress<sup>[5]</sup>, and it was never evaluated in this experimental model before.

In sum, we believe that the results demonstrated in the present study are directly connected, and explain itself by observing the studied pathway. We don't agree that the manuscript should be changed to a shot report, due to the original results presented in this study. Is true that our group has published a previous study that demonstrated the initial hypothesis of NAC effect on PPVL. The present study is a much more elaborated line of reasoning in which we demonstrated that the effects on the antioxidant NAC are due to its modulation on the eNOS-VEGF pathway.

The spelling on the article was revised by a translation service of Hospital de Clínicas de Porto Alegre, and we have attached the letter to your knowledge.

We added the units missing in the abstract, but we couldn't increase it due to the magazine's rules as for the number of words.

We sent the editor another version of our graphics in order to best distinguish them from one group to another and rewrite the group comparison according to yours and the editor's suggestion. The portal pressure result demonstrated comparison between PPVL vs SO (<sup>a</sup>) and PPVL + NAC vs PPVL (<sup>b</sup>). We added this data to the graphic. As for the rest of the results, they stand only for "a", since it is the comparison of one group (PPVL) against all the other ones, so there is only one indicative of comparison.

We decided not to extend the Background and results due to the size the article would be. We believe it would make the article much extensive.

We deeply appreciate your attention to revise our manuscript.

### 3. Reviewer 03055599

The authors conducted a brief study: “N-acetylcysteine modulates angiogenesis, vasodilation, and DNA damage in portal hypertensive rats”. In my opinion, authors must specify in the title the organ studied; “N-acetylcysteine modulates angiogenesis, vasodilation, and DNA damage in of portal hypertensive rats”; authors do not mentioned that the all experiments were carried out in stomach. The phrase in introduction: “The reactive oxygen species that characterize oxidative stress have the potential to bind to proteins, break DNA, and induce cell damage by interactions with various cell components. This phenomenon is associated with a series of disorders, including PH [15]”. The authors should explain the relationship between PH and reactive oxygen species (ROS); it is known that in the PPVL model, ROS is not increased in all organs. The author should explain this. Moreover, author do not explained the importance of ammonia level in PH. It is believed and it is general accepted that ammonia is the responsible of ROS in PH. In the section Material and Methods, the reference 19 when authors explain the surgery process. I think that the proper reference for the PPVL is Vorobioff J. et.al; 1983. When authors explained the immunohistochemistry process they mentioned the slides but never mentioned the thickness of them. Why authors did not perform perfusion in the animals? It is known that perfusion is the best method for tissue studies. If author used the same animals for IHC and WB they should explain that the samples were divided in two and that was processed in different ways. The results are written very short. Why authors did not performed the NTT WB? In my opinion the comet assay should be the second mentioned results because is a kind of control of NAC action. In general, numbering of references is not correct

We added the studied organs in the title of the article (blood and stomach).

In the phrase pointed by the reviewer, we believe that the relationship between the ROS and PH is explained in the anterior phrase “*The role of oxidative stress in the vascular dysfunction of PH has been well established in the literature*<sup>[12, 13]</sup>. The superoxide anion radical ( $O_2^{\cdot -}$ ) can react with nitric oxide to form peroxynitrite ( $ONOO^-$ ) and contribute to an increase in oxidative phenomena<sup>[14]</sup>”.

The direct way that reactive species of oxygen is related to PH is due to the overproduction of nitric oxide<sup>[2]</sup>, that reacts to ( $O_2^{\cdot -}$ ) and improves the oxidative damage<sup>[4]</sup>. We improved the paragraph in order to best clarify this statement, and highlighted in the edited-version of the manuscript.

The experimental model of PPVL is the best one to study the alterations in the systemic and splanchnic circulation related to PHT<sup>[6]</sup> and it is widely used to study portal hypertensive gastropathy. Our group has use it to study the stomach of portal hypertensive animals in several studies<sup>[1,7,8,9]</sup>.

The overproduction of nitric oxide is a damage related to the development of hyperdynamic circulation in portal hypertension<sup>[2]</sup>. The development of this collateral circulation facilitates the development of the portal-hypertensive gastropathy, leading to stomach damage. We added a phrase in the first paragraph of the discussion in order to clarify that, and the phrase “*Vasodilation and the formation of portosystemic collaterals contribute to increased blood flow and worsen PH, increasing the risk of upper gastrointestinal bleeding*” at the introduction reaffirms that.

We did not mentioned the ammonia levels at the present study because the experimental model used (PPVL) does not inflict liver damage, or cirrhosis<sup>[6]</sup>. So the PPVL model is used to study the vascular abnormalities at the PH, not the damage inflicted by a cirrhotic liver.

According to many studies published by our group, using the PPVL model<sup>[1,7,8,9]</sup>, we always cited the reference Sikuller, 1985, since he was the one to standardize the partial portal vein ligation, and it is based on his article that we performed the surgery in our animals. This explanation goes to why we did not used perfusion in this work.

Moreover, in all the studies conducted by our laboratory in several organs (stomach <sup>[1,7,8,9]</sup>, intestine <sup>[10,11,12]</sup>, liver<sup>[13,14]</sup>) we also studied oxidative stress and its patterns, and never we performed the perfusion technique.

We added the thickness of the slides in the Material and methods section at the edited-manuscript, and also the procedures for the sample's separation.

We decided not to extend the results due to the size the article would be.

As for the NTT WB, we tried to perform the same procedures described in reference articles, but the NTT expression is very hard to appear in the *Western blot* technique. As the IHC is a very specific technique, we decided to keep it in the article in order to demonstrate the presence/absence of it in our samples.

We decided to keep the comet assay as the last result because the whole article is discussed relating the results in the order as they are related to each other. So changing it would not be good for the article organization.

We revised the references according to yours and the editor's suggestions.

We deeply appreciate your attention to revise our manuscript.

## References

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Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

A handwritten signature in blue ink that reads 'Francielli Licks'.

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