April 23, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 9260-review.doc).

Title: Gastric expression of NADPH-oxidase, inducible nitric oxide synthase and myeloperoxidase in relation to nitrotyrosine in Helicobacter pylori-infected humans

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Name of Journal: *World Journal of Gastrointestinal Pathophysiology*

ESPS Manuscript NO: 9260

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

2.Revision has been made according to the editor´s suggestions in the article. All revisions made are marked in red in the text.

3 Revision has been made according to the suggestions of the reviewers
Reviewer 02441485

a) The manuscript should be improved for English language:
English language has been improved in the manuscript

b) Why did you perform gastric biopsy in patients who do not seem to need it? In fact, in paragraph Study groups (page 4, from line 25) you report only 7 patients with symptoms
Healthy subjects volunteered to esophagogastric endoscopy with mucosal biopsy takings. The majority of the volunteers had no history of gastrointestinal disease, however as described in the text four individuals in the negative, and one in the positive group had gastroesophageal reflux, and two individuals in the positive group had developed a duodenal ulcer. The study persons were not patients, but volunteers. Findings made at gastroscopy, such as signs of gastroesophageal reflux and ulcer did not exclude individuals from the study.

c) Results obtained with anti-MPO and anti-nitrotyrosine are a bit 'questionable as poorly suited for quantification of protein expression. Indeed they identify more than one band, at different molecular weights. The ideal choice would be to use an ELISA assay.
The available antibodies used to detect MPO and nitrated proteins (anti-nitrotyrosine) are well described and validated for quantification of proteinexpression. The anti-myeloperoxidase rabbit antibody used detects MPO subunits at 12 and 60 kDa. In the study we used both bands for identification, and got similar results. However the quantification presented in results is from the 60 kDa band. This fact is now described and clearyfied in Materials and Methods as follows "In the present study the 60 kDa band was used for quantification of the protein". The anti-nitrotyrosine rabbit antibody used to assess nitrated proteins identifies proteins att different molecular weights. We used several quantification methods to minimise the risk of false negative results, but we did not reach any significant differences at any case. The band described in fig 5 is the 66 kDa band of nitrated proteins, and is one of the validated and described bands detected by the positive control.
The use of ELISA is an excellent recommendation, and could be an alternative method, but in this study we have used the Western blot method with reliable results.

d) Furthermore, there is a large gap in the WB method, is not minimally mentioned the use of an antibody for

the detection of housekeeping proteins (such as b-actin or GAPDH) essential for the normalization of the amount of protein for each lane.
This is an important comment. In our group we are currently using mainly GAPDH as housekeeping protein for normalization of the amount of protein for each lane. As described in the text under Materials and Methods other positive controls were used in the present study. For iNOS the positive control used was RAW 264.7 (sc 2212, Santa Cruz), for MPO and NADPH-oxidase HL60 was used as positive control, and for nitrotyrosine the nitrotyrosine immunoblotting control (12-354, Upstate) was used.
The limited number of individuals studied enabled us to run all samples for one protein together minimizing the risk of false results.

e) For each studied protein, it is necessary to show a representative picture of WB analysis in the Results.
Representative picture of WB analysis for each studied protein is now added to the article as figure 5

f) The Abstract is well written and complete, there are only a few minor criticisms: In the first line of Background paragraph, please report the name of the bacterium in full: "Helicobacter pylori" instead of "H. pylori".
Text is changed to *"The study was undertaken to investigate the relationship between Helicobacter pylori and the oxygen and nitrogen radical system in humans.*

g) In Results paragraph in the abstract, please report the number of individuals who were found to be H. pylori positive.
The following sentence is added to the abstract "The individuals who undervent gastroscopy were divided in a H. pylori negative (n=13, mean age=39, f/m= 6/7) and a H. pylori positive group (n=8, mean age=53, f/m=3/5)."

h) Abstract.Always in the results, I suggest to emphasize the fact that the differences are significant.
Changes are made in the text in the abstract. P-values is added to the results in the abstract.

i) Background 1) Page 4, lines 3-7. "A recent study suggests the H.pylori HP0013 protein to be an enzymatic NO detoxifying system for the in vivo microbial protection ……. Several pathways have been described to inhibit the iNOS by suppressing the activation of NADPH oxidase and Jak2/Stat3 in ……." These two sentences are not so clear. Please rephrase.
Text is changed to "A recent study suggests that H.pylori contain specific proteins enabling the pathogen to cope with the damaging effects of NO. These systems are suggested to be a part in the microbial protection against nitrosative stress" and "Several traditional anti-inflammatory drugs have been shown to inhibit the induction of iNOS by suppressing the activation of NADPH oxidase in Helicobacter pylori-infected gastric epithelial cells"

j) Results 1) It is preferable to fully write "MPO" when used as paragraph title (page 7, line 3).
Text changed to "Myeloperoxidase"

k) Results 2) Page 7, lines 10-12. The following sentences "The expression of NADPH-oxidase was analysed by detecting the NADPH-oxidase subunit p47-phox expression. During activation of NADPH-oxidase, p47-phox migrate to the plasma membrane where it associates with other subunits to form the active complex." are best suited for M & M section or at most for Discussion.
The following sentence is moved to Materials and Methods: "During activation of NADPH-oxidase, p47-phox migrate to the plasma membrane where it associates with other subunits to form the active complex."

l) Discussion 1) It seems to me improper to write "production of nitrotyrosine," Perhaps it is better to refer to "proteins containing nitrotyrosine"
Text changed to "proteins containing nitrotyrosine"

m) Discussion 2) Page 10, lines 5-8. “The present study does not provide data on if H. pylori also inhibit the oxy-radical formating enzymes. However it is shown that H. pylori ……. Catalase and arginase are other examples of antioxidant proteins …….”. This paragraph is not clear in this context.

Text is changed to be more clear: “The present study does not provide data on if H. pylori also inhibit the oxy-radical formating enzymes. Oxidative stress could potentially have a negative effect on the ability of H. pylori to colonize the human stomach. However it is shown that H. pylori produces a number of antioxidative proteins, the most described ones being bacterial produced superoxide dismutase (SOD) [29]. SOD production is described as being important for the growth and survival of H. pylori under conditions of oxidative stress, and is regarded as a virulence factor affecting the ability of the microbe to colonize the stomach. Catalase and arginase are other examples of antioxidant proteins produced by H. pylori that might contribute to bacterial survival under conditions of oxidative stress[23,30,31].

Reviewer 02444986

a) The number of case and controls are too few for statistical analysis. The controls must be sex and aged matched. but H. pylori group is younger and female dominant.
This is a very important comment, pointing at the importance of not presenting false positive or false negative results. There are no statistical differences between the groups regarding age or sex. Biopsies were taken from volunteers, and the H.pylori status was not diagnosed before the endoscopy. It is always preferable to include as many individuals as possible to reach higher significance. However in this study number of individuals studied was enough to perform statistical analysis.

b) Grafics should be scattere-plot instead of box-plot.
Graphics is now changed into scatter-plot in figure 1-4

c) Grammar of some sentences should be corrected i.e “abstract conclusion: Expression of iNOS, MPO and NADPH-oxidase was up-regulated in the antrum of the H. pylori infected group. Regarding nitrotyrosine formation, Western blot did not show any significant change compared to controls.”“introduction: Untill few years ago little was known about how the H. pylori could avoid being eliminated by the acute host defence and establish a chronic infection in the human gastric mucosa. It is found that H. pylori interferes with reactive oxygene species (ROS) such as superoxide anion (O2-) that plays an important role in the elimination of invading microorganisms.”
Text changed in Abstract conclusion: “iNOS, MPO and NADPH-oxidase was up-regulated among H. pylori infected. Regarding nitrotyrosine no difference was found. This support an H. pylori related inhibition of radical formation.”
Text changed in Background “Until few years ago little was known about how the H. pylori could avoid being eliminated by the acute host defence and establish a chronic infection in the human gastric mucosa. It is found that H. pylori interferes with reactive oxygen species (ROS) such as superoxide anion (O2-) that plays an important role in the elimination of invading microorganisms.

Reviewer 02444931

a) Authors should show the results of Western blots.
Representative picture of WB analysis for each studied protein is now added to the article as figure 5

b) The results is too simple.author should add some logic analysis
The following text presented in results now includes calculations on mean + SD for all proteins, and description on Western blot bands.

iNOS:*Using western blot analysis iNOS was detected as a 130 kDa band. The iNOS expression was upregulated*

in the antral region of H. pylori infected subjects compared to the non-infected controls as shown in Figure 1, mean ± SD being 12.6±2.4 vs 8.3±3.1, p<0.01. Typical Western blot for iNOS with a band at the 130 kDa in the positive control RAW 264.7, and in human antral mucosa retrieved from H. pylori positive (HP+) and H. pylori negative (HP-) volunteers during endoscopy is shown in figure 5.

Myeloperoxidase:*As shown in Figure 2, MPO expression was markedly upregulated in the antral region of the H. pylori infected subjects compared to the non-infected controls, mean ± SD being 5.1±3.4 vs 2.1±1.9, p<0.05. In several of the non-infected controls it was not possible to detect any MPO expression at all, whereas the expression was high in all the infected subjects. Western blot of the MPO positive band at 60 kDa in the positive HL60 control and in gastric mucosal samples of HP+ and HP- volunteers is shown in figure 5.*

NADPH-oxidase:*The expression of NADPH-oxidase was analysed by detecting the NADPH-oxidase subunit p47-phox expression. P47-phox was detected as a 47 kDa band using Western blot. Figure 3 shows a significantly higher expression of p47-phox in the antral region of the H. pylori infected subjects compared to the non-infected controls, mean ± SD being 3.1±2.2 vs 0.3±0.2, p<0.01. P47-phox was low in all non-infected controls. In the H. pylori infected subjects there was a large spreading of the p47-phox expression. A typical Western blot result is shown in figure 5.*

Nitrotyrosine:*Western blot analysis did not show any significant increase nor decrease in nitrotyrosine formation in the antral region of the infected subjects compared to non-infected controls, 7.0±0.9 vs 6.9±1.1, not significant (Figure 4). Regarding Western blot representing Nitrotyrosine, several bands of Nitrated proteins could be analysed. Shown in the figure 5 is a typical band at 66 kDa in the positive control and in HP+ and HP- samples.*

c) Some grammar errors should be corrected. Such as neutrofils and macrofages should be placed by neutrophils and macrophages. in vivo in background section should be in vivo (italic)

Neutrofils and macrofages are replaced by neutrophils and macrophages.

In vivo *is replaced by in vivo (italic)*

4 References and typesetting were corrected

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

Sincerely yours,

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