ANSWERING REVIEWERS

February 11, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 7509-review.doc).

Title: Involvement of heat shock proteins in gluten-sensitive enteropathy

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Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 7509

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

1 English language editing was performed by an editing company.

2 Revision has been made according to the suggestions of the reviewer:

(1) Reviewer No. 00187623

The conclusion is exceptional in its summarization of the practical aspects of the topic. The current use of the Heat Shock Proteins might be discussed a bit more. Nevertheless, I see this as a good resource for investigators looking at this topic. I would suggest accepting the article with the following changes: On page 2: The incidence of CD continued to increase worldwide in the past decade however most cases still remains undiagnosed-- this should read "still remain undiagnosed", On page 3: — including inflammatory bowel disease (IBD), allergy or asthma — beside of genetic predisposition environmental factors has a crucial impact. "this needs revision - unclear" . In conclusions, consider elaborating further or more specifically regarding the potential benefits of evaluating the proteins.

Answer: Thank you for the reviewer's precious comments, we expanded the manuscript and discussed further the potential benefits of HSPs. The suggested modifications were performed.

(2) Reviewer No. 00002649

1. In Figure 1, authors summarize the key processes during the pathogenesis of CD. They do not label gliadin. They also indicate in the text that gliadin peptides can directly activate TLRs on macrophages and DCs, which leads to the upregulation of proinflammatory cytokiens and chemokines, which is not shown in Figure 1. TLR's are also not shown. These should be added to Figure1. Is TG the same thing as TTG?

Answer: According to the suggestions of the reviewer we modified Figure 1 and incorporated the missing parts including TLR receptors and the direct effects of gliadin peptides on DC-s and macrophages. TG is the same that tTG meaning tissue transglutaminase. We specified it in Figure 1 and in the figure legend as well.

2. On page 8, Inflammation and HSPs, the first sentence should be deleted because CD is not the topic of the section. Also, the authors summarize that HSP60 stimulates DCs more rapidly than LPS; and HSP-peptide complexes bind to TLR2 and 4 on the surface of APC and activate the T cell pathways

leading to enhanced expression of proinflammatory cytokines. On the other hand, HSP60 resolves inflammation by up-regulation of Tregs. These findings seem opposite -- how do the authors explain this?

Answer: As suggested the first sentence was deleted. We are thankful for the comment, and we revised this part of the manuscript. In contrast to the known function of HSP60 as a "danger signal" for the immune system via the activation of TLR 4 on the surface of innate immune cells, it was recently demonstrated that HSP60 can also contribute to the resolution of inflammation when acting on TLR 2 on T cells. These data suggest that the immunmodulatory effect of HSPs can be cell and receptor type specific.

3. The authors state on p. 9 "HSP60 has been shown to be a novel mitochondrial permeability transition regulator.." Could they give a little bit more detail on this? How does HSP60 regulate mitochondrial permeability and apoptosis?

Answer: Thank you for the remarks. More detailed information was added about the regulatory role of HSP60 in mitochondrial permeability.

4. On page 11, lines 7-10, the authors promote the concept that activation of TLR signaling produces pro-inflammatory cytokines and inflammation with further disruption of barrier function. However, HSP70 activates TLR2 and TLR4 which "can contribute to the maintenance of intestinal barrier function by preserving the integrity of the tight junction proteins..."?

Answer: Thank you for your remark. As demonstrated by Zhanin et al. TLR2 and TLR4 activation can contribute to both induction and termination of effector immune responses [1]. Since HSPs can exert their immunomodulatory effects also through different TLRs found on different cell types, this may explain the apparent discrepancy.

1. Zanin-Zhorov A, Cohen IR. Signaling via TLR2 and TLR4 Directly Down-Regulates T Cell Effector Functions: The Regulatory Face of Danger Signals. Front Immunol. 2013 Jul 25;4:211. doi: 10.3389/fimmu.2013.00211.

5. The summary gives some evidence that HSP induction may be used to treat CD. This paragraph should be expanded. Injury in animals produced by NSAIDs, dextran sulfate colitis, and bisphosphonate induced cell injury are prevented by the HSP inducer geranylgeranylacetone. Do the authors postulate that such drugs or vaccines will eventually replace the gluten-free diet?

Answer: As suggested by the reviewer the manuscript was expanded containing further information about the beneficial effects of HSPs as potential therapeutic targets. Thus a new chapter with the title "HSPs and therapeutic treatments" was added. However HSP-inducers are promising drugs to treat gastrointestinal diseases, further studies are needed to test their effects on the pathophysiology of CD to postulate whether they can complete or replace gluten-free diet. But there is no doubt that the reviewer's question is relevant and it would be beneficial to answer it in the future.

Minor comments:

1. The authors use "pathomechanism" throughout. I am not familiar with this word. Do they mean "pathophysiology" or "pathogenesis" or "disease mechanism"?

Answer: We agree with the reviewer and according to the suggestion we defined it throughout the manuscript and wrote "pathophysiology" instead of "pathomechanism".

2. INTRODUCTION: -Ninety-five percent should be spelled out when it is the first word of the sentence. -Second paragraph, second sentence: "asthma – a genetic predisposition combined with environmental factors leading to disease manifestation."-Under "Effect of stress…" would replace "beneficial" with "compensatory."-"homeostasis and ensuring survival."-(such as heat, toxins, radiation, infection, mechanical force, and metabolic disturbances." 3. Page 4: Would delete "therefore exerting an immunomodulatory activity." 4. Page 4: "Reaching the epithelial cells, gluten enhances IL-15…" should be placed after reference 16 because it is the initial event before gluten residues reach

the lamina propria.

Answer: According to the reviewer's comment, all of the above suggested modifications were performed in the manuscript.

5. What is meant by "organisatory"--?should it be deleted? 6. Top of p. 6, first sentence, would include intestinal and colonic epithelium. 7. Page 7, would reverse the 1st sentence to say "Several environmental and chemical agents inducing oxidative stress may lead to enhanced ROS…" 8. Second para under Oxidative stress: Wischmeyer and Chang also showed in vivo that induction of hsp (by glutamine feeding) preserved intestinal epithelial cells. So there are studies in vitro (cited) and in vivo.

Answer: As suggested by the reviewer, the findings of the *in vivo* study were incorporated into the manuscript and a citation was added to the reference list. All of the above proposed modifications were made.

9. Page 8, under Inflammation and HSP's: the conclusion sentence can be part of the paragraph above. 10. Page 9: I would change the topic from Mucosal Damage and HSPs to Intestinal Epithelial Integrity and HSPs. 11. Page 10: All grammar issues, because the section is critical. First sentence "not surprising, in part owing to the lack of experimental models which makes it challenging to identify the complex pathophysiology of CD." HSP are present throughout the GI tract; however, there are… In contrast, small intestinal expression of HSPs is normally negligible, but under stress, HSP25 and HSP70 are markedly increased. The applied gluten free diet reduced clinical sx and also the level of intestinal HSP72, but its expression remained elevated…"We also demonstrated that the most abundant expression of HSP72 was in villus enterocytes and immune cells of the LP…" 12. Page 11: Would delete "there are only a few data available—because this might irritate some investigators, e.g. Chang E et al. "Previously, Yang et al found that heat stress increased protein transport… What is meant by protein transport?

Answer: The suggested sentence was deleted and a clarification for protein transport was added meaning the transport of horseradish peroxidase to evaluate human intestinal epithelial permeability.

13. "strengthened by Cario et al (98), who reported that…" 14. Suggest: "Other HSPs such as HSP65 and small MW HSPs (<30KD) were also suggested to contribute to the pathophysiology of CD." 15. Would not state "excluded for the disease" even though this was published. The authors I think mean "without CD based on a normal biopsy." 16. Page 11, bottom: What is meant by "slow" and "fast"?

Answer: The adjectives "slow, fast and intermediate" refer to the electrophoretic mobility of the DNA. As suggested it was clarified in the manuscript.

17. Is IBD spelled out the first time? 18. Would delete "good" bacteria or say "beneficial" 19. Table 2. The authors need to put a statement in the MS such as "Table 2, below, summarizes…" In this table, I think the T84 cell studies should be deleted because CD is not part of those reports.

Answer: As suggested by the reviewer the data was removed from Table 2.

Also, "excluded from having the disease" rather than "excluded for…" 20. SUMMARY: "In this review, we summarize the main…" "chaperones" sp. Needs a comma after CD in the first paragraph and after factor in the second. The second and 3rd paragraphs can be combined.

Answer: We are grateful for the reviewer's precious comments. We modified the manuscript according to all of the above suggestions.

(3) Reviewer No. 00001391

The major point is: increase expression of HSP is a biomarker or simply a consequence of the immunotoxic agression of gluten in CD or predisposition to CD patients. I think you have to focus on this point.

Answer: Thank you for the precious comment of the reviewer. Based on the findings in CD, it can not be excluded that HSPs may serve as biomarkers. HSPs do not seem to be specific for CD, but their presence indicates stress-induced responses and their increased expression may reflect disease activity. To evaluate the significance of HSPs as markers, comprehensive human studies are needed.

1. Abstract: there is no relation, at the present time, between new possible CD treatment and HSP. So delete or precise in the abstract. The review is not "recent". I think it is the "present" review.

Answer: According to the suggestion of the reviewer the abstract was revised.

2. Page 4 Anti-gluten is "anti gliadine" antibodies?

Answer: Thank you for the comment. They are anti-gliadin antibodies, thus we defined it in the manuscript.

3. Page 5. Can you precise "nutritional" stress/ors?

Answer: Based on the findings of Siddiqui et al. nutritional stresses, specifically the absence of glucose and glutamine, can induce the expression of heat shock proteins [1]. According to the reviewer's suggestion we modified the manuscript and a reference was added to the reference list.

2. Siddiqui F, Avery PR, Li CY, Zhang X, LaRue SM, Dewhirst MW, Ullrich RL. Induction of the promoter HSP70B by nutritional stress: implications for cancer gene therapy. Cancer Invest. 2008; 26(6):553-61. doi: 10.1080/07357900701788015.

4. Page 6 Can you precise type of tissue for HSP family especially if intestine is involved

Answer: HSPs were shown to be predominantly localized in the intestinal epithelium (both in duodenal and colonic epithelium) [2, 3]. Investigating the localization of HSP72 in patients with CD we found that it is expressed not only in villous enterocytes but also in the lamina propria [3]. We defined it in the manuscript and in table 2.

3. Kojima K, Musch MW, Ren H, Boone DL, Hendrickson BA, Ma A, Chang EB. Enteric flora and lymphocyte-derived cytokines determine expression of heat shock proteins in mouse colonic epithelial cells. Gastroenterology 2003; 124(5): 1395-1407

4. Sziksz E, Veres G, Vannay A, Prokai A, Gal K, Onody A, Korponay-Szabo IR, Reusz G, Szabo A, Tulassay T, Arato A, Szebeni B. Increased heat shock protein 72 expression in celiac disease. Journal of pediatric gastroenterology and nutrition 2010; 51(5): 573-578. DOI: 10.1097/MPG.0b013e3181ea0092

5. Page 12 I think it is not necessary to have details concerning IBD, a very different pathology to my view

Answer: We agree with the reviewer that the pathology of inflammatory bowel disease (IBD) and celiac disease (CD) is different, however there are some overlaps in their pathophysiology. In both diseases the antigen induced inflammatory pathways lead to the impairment of tight junctions causing increased permeability of the intestinal epithelium and extensive damage of the intestinal mucosa [4]. Based on these similarities and because of the lack of an experimental animal model of CD, which could ensure the exact examination of HSPs, we decided to take also a short outlook to the role of HSPs in IBD, however according to the reviewer's suggestion we shortened this part of the manuscript.

5. Festen EA, Szperl AM, Weersma RK, Wijmenga C, Wapenaar MC. Inflammatory bowel disease and celiac disease: overlaps in the pathology and genetics, and their potential drug targets. Endocr Metab Immune Disord Drug Targets. 2009;9(2):199-218.

6 What kind of therapeutic modulation can you speculate with HSP and how in CD?

Answer: Thank you for the remark, according to the suggestion of the reviewer we incorporated a

new chapter about the potential therapeutic benefits of HSPs into the manuscript.

3 Format has been updated.

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

Sincerely yours,

Sziksz Erna

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