Author's response to reviews

Title: Anti-inflammatory activity of edible oyster mushroom is mediated through the inhibition of NF-kB and AP-1 signaling.

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Author's response to reviews:

February 27, 2011
Dr. Nehme Gabriel
Editor-in-Chief
Nutrition Journal

RE: MS 1053030903468549

Dear Dr. Gabriel,

Please find enclosed our revised manuscript entitled “Anti-inflammatory activity of edible oyster mushroom is mediated through the inhibition of NF-kB and AP-1 signaling.” by Andrej Jedinak, Shailesh Dudhgaonkar, Qing-li Wu, James Simon, and Daniel Sliva.

First we would like to thanks reviewers for their valuable comments. Our responses follow their comments and suggestions. All changes are underlined in our revised manuscript.

Referee nu. 1:
The number of times experiments have been done needs to be clearly described for each figure.
The effect of the OMC on viability of cells needs to be looked at in every in vitro assay exactly as the assay was done. It is not adequate as reported to add OMC alone to RAW cells and say that an increase in death was seen at the highest dose and after 48 h. The stimulation in the presence of LPS and OMC needs to be done to determine if cell death and not regulation of inflammation is responsible for the observations. Same with Fig. 5 data.

Our response:
Number of experiments with proper statistics is described in each figure in revised manuscript. We are showing new figure 1 with the data demonstrating that OMC is not toxic. In addition, LPS itself at the concentration used in our experiments is not toxic as shown in our experiments and also by other authors. The slight decrease in cell viability after 48 and 72 hours (new Fig. 1N) is not responsible for the anti-inflammatory effects of OMC because the combination of OMC with LPS did not change the morphology of treated cells (not shown). In low doses of OMC, where is the viability same as in control, significantly decreased expression and secretion of pro-inflammatory cytokines and mediators. In addition, we are further confirming the molecular mechanism of this anti-inflammatory activity of OMC.

Referee nu. 1:
The claim that OMC suppressed endotoxemia is not supported by the data. IL-6 did not change and although TNF-a decreased significantly it is not clear whether the small decrease would protect against endotoxemia.

Our response:
We agree that a significant decrease in the TNF-a and non-significant decrease in IL-6 is not probably sufficient for the claim that OMC protects against endotoxemia. Therefore, we changed the title of our manuscript, and removed our statement that OMC inhibits endotoxemia. We are presenting our data as an inhibition of inflammatory response in vivo.

Referee nu. 1:
The analyzes of the extract are puzzling. The description of the extract suggest that only water soluble molecules are used in the assays. Characterization of that fraction would be interesting. The mass spectra data will not be overly informative to the readership of J Nutr. In addition, why was an ethanol extract Fig 8 characterized. Only the water extract was used.

Our response:
We have performed an additional analysis of water extract OMC and demonstrated that OMC contains large amount of glucans. Although the mushroom beta-glucans are usually associated with the immunostimulatory activities of certain mushrooms, recent studies demonstrated that some glucans have actually anti-inflammatory effects. We have removed mass spectra from our manuscript as well as the analysis of an ethanol extract.

Referee nu. 2:
This paper investigated a suppression effect of an extract of oyster mushroom (Pleurotus ostreatus) on inflammatory response in macrophages and murine lymphocyte. This is a very interested work. Because the authors focused activities of several compounds in the mushroom. It has generally been considered that immunomodulating activities of mushrooms were brought about by functional polysaccharides such as #-glucan. However, mushrooms are food materials, and they contained many kinds of compound. Therefore, it is
necessary that bioactivities of other nutritional and functional factors as well as a polysaccharide are investigated.

However, in this works, a number of points need revising before publication. These are given below.

Major point was:

1. It is enough to investigate anti-inflammatory effect of OMC on only macrophages. Murine splenocyte stimulated with ConA was considered to be mainly a T cell. Functions of macrophages and T cell are different in immune system. In this study, relationship between them was not clear.

Our response:

We agree that the functions of macrophages and splenocytes are different in immune system. However, our point is to demonstrate that OMC modulates (inhibits) the activity/function of different targets in the immune system.

2. How did sole OMC stimulate macrophages? Did not OMC stimulate macrophages? I think that OMC also contains several immunomodulating compounds, but not?

Our response:

We were not able to detect any stimulatory effect of OMC on macrophages. In addition, our further analysis of OMC demonstrated that OMC contains alpha- and beta-glucans. Although the mushroom beta-glucans are usually associated with the immunostimulatory activities of certain mushrooms, recent studies demonstrated that some glucans have actually anti-inflammatory effects.

3. In this study, cells were treated with OMC before LPS addition, weren’t they? If it was so, was not it considered that OMC suppressed inflammatory response cascade, but inhibited to LPS binding to TLR-4?

Our response:

Thank you for an excellent point. We are currently working on the isolation of specific OMC glucans, and our future study will focus on the possible interaction of these glucans with glucan receptors TLR4 and Dectin-1. Nevertheless, our current study is the pilot study demonstrating anti-inflammatory activities of the Oyster mushroom and possible molecular mechanism through the inhibition of NF-kB and AP-1.

Minor points were:

1. Study on an anti-inflammatory effect on murine splenocyte must be deleted.

Our response:

See our response above.

2. EMSA in materials and methods:

An explanation of EMSA must be written in detail.

Our response:
EMSA is described in detail with appropriate reference.

3. In Figure 1:
I did not think that this is necessary in this work. Did not net numbers of cells increase after 48 hr?
Our response:
New Fig. 1 is included demonstrating that OMC do not inhibit the viability of RAW264.7 cells but also did not stimulate proliferation (number) of these cells.

Referee nu. 3:
The authors have well described an interesting study about anti-inflammatory effect in vitro and in vivo of oyster mushroom and, they have slightly analyzed the composition of this material. Pleorotus ostreatus is a valuable source of nutrients and bioactive compounds in addition to the growing appeal for humans by their flavors and culinary features. According to experimental and clinical findings, systemic inflammation is implicated in serious diseases. Recently, some studies have demonstrated the beneficial effect of oyster in improvement, prevention or treatment of diseases. But the implicated mechanisms are not yet clear. The publication of manuscript “Edible oyster mushroom suppresses inflammatory response in macrophages and in animal model of endotoxemia” could contribute knowledge of them.

Major Compulsory Revisions:
In general, the methods are not well described. More details would be necessary to replicate this work. In fact, I think the method would need some explanations.
It is not clear if OMC is an extract in water or lyophilized extract.
I think it enough with 1 o 2 hours, why have you incubated overnight?
Was the material stirring during incubation?
How have you prepared the extract of OMC with 100% water and 100% ethanol to analysis by LC? Since there are standards of all identified compounds by LC/MS, why have not you quantified the peaks from LC with corresponding standards?
In addition, more information about analysis in vivo and in vitro must be added.
The discussion must been reviewed and you must include and compare more studies about the anti-inflammatory effects of Pleorotus sp. and other mushrooms especially edible mushrooms.
Our response:
The methods used in our study are described in details in our revised manuscript, as suggested by the reviewer. Specifically, the preparation and analysis of OMC is described as well as new analysis demonstrating that the major components of OMC are glucans. Our discussion is also expanded with the comparison of other studies demonstrating anti-inflammatory activities of Pleurotus and other edible mushrooms.
Minor Essential Revisions:

Background, second paragraph: The reference of Kaplanski G et al. [4] is a review about IL6 and its roles in inflammation but in this have not been cited any study about medicinal or edible mushrooms. In my opinion, this citation is inappropriate for this paragraph.

Minor issues not for publication

Abstract, results, first paragraph: “Anti-inflammatory activity of OMC (was confirmed by the inhibition…”

Remove “(“.

Methods, Cells, first paragraph: 37oC instead of 370C. But you must review the temperature units of this manuscript because there are a lot of errors about degrees centigrade.

Methods, Western blot analysis, first paragraph: You must write “μg” without underline.

Methods, Electrophoretic mobility shift assay: This paragraph must be left and right justified.

Methods, Densitometric analysis: Use capital letter for HP scanjet.

Methods, Analysis of OMC, first paragraph: In the text “nebulizer at a flow rate of 12 L/Min,…)” fist letter of minute (M) must be write with small letter.

Methods, Statistical Analysis, first paragraph: “Student t-test” must be change to “Student`s t-test”.

Results, OMC suppresses LPS-dependent induction of cytokines and inflammatory mediators in macrophages, second paragraph, second line: you must change the comma to a point after “PGE2 and NO”.

Results, Effect of OMC on the LPS-dependent induction of transcription activity of AP-1, NF-KB and STAT3, first paragraph: You must use EMSA instead of GEMSA.

Results, OMC possesses immunosuppressive activity, first paragraph: “We found that OMC markedly suppressed Con-A-dependent production of IFN-# and IL-2, respectively, suggesting that OMC…” I think the word “respectively” is used incorrectly in this sentence.

Figure legends, Figure 3: You must use EMSA instead of GEMSA.

Figure legends, Figure 4: You must include the comma after “means±S.D.”.

Figure legends, Figure 5: You must write “μg” without underline.

Figure legends, Figure 8: the peak 1 has been omitted, why?

Table 1: In its legend you must indicate that these data are “(mean±S.D)”. Our response:

All suggested corrections are included in our revised manuscript.
Thank you for your consideration.
Yours sincerely,
Daniel Sliva, Ph.D.
Cancer Research Laboratory
Methodist Research Institute
Indiana University Health