Veterinaria México OA
Publicación Digital de la Facultad de Medicina Veterinaria y Zootecnia
Universidad Nacional Autónoma de México | FMVZ

Submission 1195. Peer-review process

Reviewer A

Round 1

1. Does the paper comply with the scientific quality standards of its field?*

- ○ Yes
- ◉ No

2. Are the methods described in detail, so that the experiments are reproducible?*

- ◉ Yes
- ○ No

3. Is the statistical analysis of the data appropriate and technically sound?*

- ◉ Yes
- ○ No

3.1 If necessary, please, provide a brief explanation of your evaluation of the statistical methods used in the manuscript.

4. Are the claims/statements/conclusions fully supported by results?*

- ○ Yes
- ○ No
- ◉ Partially

4.1 If not, please, indicate the additional evidence that would help accomplishing this requirement.

the work is very poor without the contribution of each of the proteins identified in macrophage apoptosis

5. Are the claims/statements discussed rigorously, in the context of previous scientific literature and knowledge of the field? *

- ○ Yes
- ◉ No

6. If your recommended decision is to reject the manuscript in its actual version, is the study promising enough as to encourage the authors to re-submit after a major revision?*

- ◉ Yes
- ○ No

7. Is there any ethical concern related to experimental subjects (i. e. animals, humans)?*

- ○ Yes
- ◉ No

8. Is there any evidence of manipulation of figures or images that compromise the scientific quality or reliability of the paper?*

- ○ Yes
- ◉ No

9. Is the paper clearly written, following standard scientific English?*

- ○ Yes
- ◉ No

10. Have the authors made all experimental data fully available to readers?
This requirement can be accomplished through either supplementary files or by depositing data on public repositories.*

- ◉ Yes
- ○ No

11. Based on paper content and your global appraisal of it, indicate the type of publication that best fits the manuscript.

- ○ Original research article
- ◉ Short communication
- ○ Review

12. I agree to review the updated version of the manuscript.*

- ◉ Yes
- ○ No

13. Comments to the editor. Please, include a brief report stating your general appraisal of the paper. Provide the reasons that support your recommended decision of acceptance, rejection or downgrading the paper to a research note.

The submitted manuscript is in Spanish and the authors did not take care of details in the presentation of the article, as indicated in the specific comments that are indicated to them.
I suggest the authors resubmit the manuscript with the characterization of the individual proteins on the apoptosis of macrophages, to gain relevance of this study, otherwise a short communication can be considered.

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14. Comments to authors (optional). Please, provide a constructive and thorough review of the sections. So, that the authors are able to prepare a revision ready for acceptance without incurring in multiple revision rounds.

This work presents three Mycobacterium bovis proteins, which are contained in a fraction of the Mycobacterium, and which are reported together to be responsible for inducing apoptosis in bovine macrophages independently of caspases.

The manuscript follows a very similar strategy to assess apoptosis independent of caspase 3 activation as the article published in the same journal in 2019 by Maciel Rivera et al. The only originality of the manuscript presented is the characterization of three M. bovis proteins by liquid chromatography-mass spectrometry (LC-MS) that induces apoptosis independently of caspase 3, however, although the authors accept that it is a limitation of their study, it would have been a good contribution to have tested its individual activity and not together.

Specific comments.

In the summary in the contribution part (line 18), the way it is written suggests that the three proteins induce apoptosis, but since they were not evaluated individually, it is better to change the wording in a similar way as it is presented in the summary "the presence of identified the presence of three candidates (MPB70, MPB83 and the 60 kDa Chaperonin) responsible for the biological activity

In line 50 of the introduction, indicate to which species of Mycobacteria the mentioned proteins belong and on macrophages of which origin they were evaluated.

In the methodology (lines 88 to 91) it is not clear why fractions were dialyzed with water during the day and with PBS at night, clear it up

On lines 105-106 it is mentioned that "Monocytes were obtained according to the previously described method from peripheral blood of healthy bovines" Have the bovines used, even though they are healthy, had previous contact with M. bovis? If yes, discuss whether this would have any effect on the results obtained, given the reports of trained immunity that has been observed in macrophages.

Line 123 establishes that the macrophages were incubated with 100 ug of each of the fractions. How was this amount determined? And is it 100 ug per mL or per well? (specify).

In line 136, it is mentioned that the cells were incubated with the DNA labeling solution (specify what the labeling solution contains?). In the same sense, specify on line 150 what the reaction buffer contains.

In the results section, it is not clear which peaks were pooled to generate the fractions, and if in the figure, P refers to peak or to fraction? In the same results, include a clearer figure 1A, because it is not appreciated what the legends, green, red, etc., correspond to. And specify the legends of the X and Y axes. In Figure 1B, the legend is incorrect, modify according to what Figure 1B indicates.

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Include as a Supplementary Figure the analysis strategy to determine the % of DNA fragmentation.

In the legend of Figure 2 (line 214) it is mentioned "in bovine macrophages stimulated with 100 µg/106 of the fraction", to clarify that they are cells (100 ug/106 cells). In that same legend, indicate with respect to which groups the comparison is being made, for the two P that they establish.

In Figure 3, the blue arrow does not include the 56.72 Da protein

Table 1 is not referred to in the text

In the discussion, line 282, according to the table included, GroEl refers to the gene, not the protein, and from the way they present the text that interacts with annexin 2, it seems that they are referring to the function of the protein.

Add the appropriate reference for following statement "there is evidence that hsp60 together with hsp10 are involved in signaling caspase 3 activation, as well as the association of these molecules with Bax, resulting in the activation of apoptosis (lines 289- 291)

In the discussion part, it is necessary that the authors point out what is the importance of inducing apoptosis independent of Caspase 3? And within the framework of the infection (host-mycobacteria relationship), point out what relevance does this work have?

As previously pointed out, the work remains very poor without the contribution of each of the proteins identified on macrophage apoptosis. Thus, authors are required to resubmit the manuscritp with the evaluation of the role of each proteín on macrophage apoptosis (to be considered as an original research manuscript), and to present the mansucript in the english format requested by the journal.

Peer-reviewed comments

Completed: 2023-05-22 10:11 AM
Recommendation: Revisions Required

Section Editor recommendation:
2023-05-31 09:40
The recommendation regarding the submission to Veterinaria México OA, "Mycobacterium bovis CFE proteins induces apoptosis in a caspase independent manner: Proteínas de Mycobacterium bovis inducen apoptosis" is: **Resubmit for Review**

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Editor Decision:

Itzel Nalleli Jimenez Vazquez, Clara I Espitia Pinzon , Erasmo Negrete Abascal, Alejandro Benítez Guzmán, Julio Morán, José Ángel Gutierrez Pabello :

Reviewers have commented on your submission to Veterinaria México OA, "Mycobacterium bovis CFE proteins induces apoptosis in a caspase independent manner: Proteínas de Mycobacterium bovis inducen apoptosis". Reviewers have requested revisions that should be addressed (see below) before the submission is accepted for publication. Therefore, we invite you to submit a revised version of the paper that addresses the points raised during the review process. We kindly suggest the revised version by **June 30, 2023**. If you will need more time than this to complete your revisions, please reply to this message.

Please, upload the following items in the "**Revisions**" section when submitting your revised manuscript:

- IMPORTANT-> Be sure **not** to include any data from the authors. If there is a need to update the author information/order, please include it in a new Discussion in the **Review Discussions**.

1) A rebuttal letter that responds to each point raised by reviewers. Please, upload this letter as a separate file labeled "**1195-RR1-yyyymmdd.docx**"

2) A marked-up copy of your manuscript that highlights changes made to the original version. You may use the "track changes" tool of Microsoft Word. However, make sure your name does not appear as the author of the document, to ensure the blind review process. Besides, do not include the authors and their affiliations in this document. As it will only be used for review, it should come with the title, followed by the abstract right away. Please, upload this as a separate file labeled "**1195-VCA-R1-TC-yyyymmdd.docx**"

3) An unmarked version of your revised paper without tracked changes. Please, upload this as a separate file labeled "**1195-VCA-R1-yyyymmdd.docx**"

Notice **yyyymmdd corresponds to the date when the author is submitting the revised manuscript.*

Please, do not submit your revised paper as a new submission to avoid having duplicates in the journal system. Moreover, notice that reviewers may cite specific lines of your manuscript in their comments. For your reference, the review version PDF file used by reviewers is attached to this message. Thank you for submitting your work to Veterinaria México OA.

Kind regards,

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ANSWERS TO REVIEWERS

The submitted manuscript is in Spanish and the authors did not take care of details in the presentation of the article, as indicated in the specific comments that are indicated to them.

I suggest the authors resubmit the manuscript with the characterization of the individual proteins on the apoptosis of macrophages, to gain relevance of this study, otherwise a short communication can be considered.

14. Comments to authors. Please, provide a constructive and thorough review. So, that the authors are able to prepare a revision ready for acceptance without incurring in multiple revision rounds.

Include your specific numbered comments, citing sections or line numbers (eg. L23-35) when appropriate. Do not place comments directly in the manuscript and/or upload a commented manuscript as a review report.

1. This work presents three Mycobacterium bovis proteins, which are contained in a fraction of the Mycobacterium, and which are reported together to be responsible for inducing apoptosis in bovine macrophages independently of caspases.

The manuscript follows a very similar strategy to assess apoptosis independent of caspase 3 activation as the article published in the same journal in 2019 by Maciel Rivera et al. The only originality of the manuscript presented is the characterization of three M. bovis proteins by liquid chromatography-mass spectrometry (LC-MS) that induces apoptosis independently of caspase 3, however, although the authors accept that it is a limitation of their study, it would have been a good contribution to have tested its individual activity and not together.

We thank the reviewer for the comments.

Specific comments.

2. In the summary in the contribution part (line 18), the way it is written suggests that the three proteins induce apoptosis, but since they were not evaluated individually, it is better to change the wording in a similar way as it is presented in the summary "the presence of identified the presence of three candidates (MPB70, MPB83 and the 60 kDa Chaperonin) responsible for the biological activity

Changes were made according to the reviewer suggestion.

2. In line 50 of the introduction, indicate to which species of Mycobacteria the mentioned proteins belong and on macrophages of which origin they were evaluated.

Changes were made as suggested.

3. In the methodology (lines 88 to 91) it is not clear why fractions were dialyzed with water during the day and with PBS at night, clear it up

The wording was changed to clarify the methodology on protein desalting.

4. On lines 105-106 it is mentioned that "Monocytes were obtained according to the previously described method from peripheral blood of healthy bovines" Have the bovines used, even though they are healthy, had previous contact with M. bovis? If yes, discuss whether this would have any effect on the results obtained, given the reports of trained immunity that has been observed in macrophages.

Bovine donors belong to a free tuberculosis herd. This information has been added to the text.

Line 123 establishes that the macrophages were incubated with 100 ug of each of the fractions. How was this amount determined? And is it 100 ug per mL or per well? (specify).

The protein concentration was determined by a PhD student in 2007. Since that time, we have used this concentration that works properly in our hands.

5. In line 136, it is mentioned that the cells were incubated with the DNA labeling solution (specify what the labeling solution contains?). In the same sense, specify on line 150 what the reaction buffer contains.

Labeling solution:

Reagent	ul
Reaction buffer	10
TdT Enzime	0.75
BrdUTP	8
Destiled water	31.25

TdT: Terminal deoxynucleotidyl transferase

BrdUTP: 5- Bromo-2' -deoxyuridine 5'-triphosphate

We used a commercial kit; therefore, we do not know what the components of the reaction buffer are.

6. In the results section, it is not clear which peaks were pooled to generate the fractions, and if in the figure, P refers to peak or to fraction? In the same results, include a clearer figure 1A, because it is not appreciated what the legends, green, red, etc., correspond to. And specify the legends of the X and Y axes. In Figure 1B, the legend is incorrect, modify according to what Figure 1B indicates.

P refers to each peak in the chromatogram. Each peak is composed by several fractions, in this study we selected peak number 4 to perform the experiments. Peak 4 corresponds to fractions 51 to 60 with a molecular weight between 15 and 20 kDa.

In the text, we changed the letter P to FI (fraction of interest) to name the fractions selected for the experiments. We also, included a clearer figure 1A and modified the legend in figure 1B.

7. Include as a Supplementary Figure the analysis strategy to determine the % of DNA fragmentation.

A supplementary figure was added as requested.

8. In the legend of Figure 2 (line 214) it is mentioned "in bovine macrophages stimulated with 100 µg/106 of the fraction", to clarify that they are cells (100 ug/106 cells). In that same legend, indicate with respect to which groups the comparison is being made, for the two P that they establish.

We changed the wording to clarify the paragraph as suggested by the reviewer.

9. In Figure 3, the blue arrow does not include the 56.72 Da protein. Table 1 is not referred to in the text.

An arrow pointing the 56.72 kDa protein was added.

The wording in text was modified to mention Table 1.

10. In the discussion, line 282, according to the table included, GroEl refers to the gene, not the protein, and from the way they present the text that interacts with annexin 2, it seems that they are referring to the function of the protein.

The name for 60 kDa chaperonin in the text was modified to avoid confusion between gene name and protein name.

11. Add the appropriate reference for following statement “there is evidence that hsp60 together with hsp10 are involved in signaling caspase 3 activation, as well as the association of these molecules with Bax, resulting in the activation of apoptosis (lines 289- 291)

The cited reference is correct.

12. In the discussion part, it is necessary that the authors point out what is the importance of inducing apoptosis independent of Caspase 3? And within the framework of the infection (host-mycobacteria relationship), point out what relevance does this work have?

We added the following paragraph to the discussion section:

This information is significant because most of the members of the *Mycobacterium tuberculosis* complex activate the caspases when inducing macrophage apoptosis (Mohareer, Asalla, & Banerjee, 2018). This finding may suggest that *Mycobacterium bovis* try to interfere with apoptosis by inhibiting caspase activation. However, macrophages may counterattack using a strategy to promote cell apoptosis in the absence of caspase activation. At the end, induction of apoptosis helps to control bacterial intracellular growth (Benítez-Guzmán, Arriaga-Pizano, Morán, & Gutiérrez-Pabello, 2018).

13. As previously pointed out, the work remains very poor without the contribution of each of the proteins identified on macrophage apoptosis. Thus, authors are required to resubmit the

manuscript with the evaluation of the role of each protein on macrophage apoptosis (to be considered as an original research manuscript), and to present the manuscript in the English format requested by the journal.

Respectfully, we do not agree with reviewer's point of view. As part of our research, we have demonstrated that *Mycobacterium bovis* induces apoptosis in bovine macrophages. Not only live bacteria, but also protein extracts induce apoptosis in the absence of caspase participation. Then we proposed AIF and Endo G as some of the proteins involve in macrophage cell death. The next question was related with the identification of the proteins associated to cell death. We set out an experimental design using two crude protein extracts: Culture Filtrate extract (CFE) and the Soluble Extract (SE, bacterial biomass). Both extracts promote macrophage DNA fragmentation. So, we decided to interrogate our system to identify which proteins are responsible for the DNA fragmentation. In this study, we started analyzing the different protein fractions of the CFE. We identified one fraction with the biological effect, and we decided to characterize the proteins in this fraction by electrophoresis of double dimension and LC-MS. Our results propose the presence of the candidate proteins. We agree with the reviewer that the next step is the production of the recombinant proteins to incubate them with macrophages and verify the biological activity of the proteins alone or in combination. We firmly believe that through the description of our results across the years, it is possible to see that each project adds new information which is important in the context of the pathogenesis of bovine tuberculosis.

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Section Editor recommendation: 2023-07-16 01:03 PM

Enrique Jesús Delgado Suárez, Miguel Cuevas Díaz:

The recommendation regarding the submission to Veterinaria México OA, "Mycobacterium bovis CFE proteins induces apoptosis in a caspase independent manner: Proteínas de Mycobacterium bovis inducen apoptosis" is: Accept Submission