Author’s response to reviews

Title: Cyclosporine-A Attenuates Retinal Inflammation by Inhibiting HMGB-1 Formation in Rats with Type 2 Diabetes Mellitus

Authors:

Peng Wang (luckywp2000@aliyun.com)
Fei Chen (731050214@qq.com)
Xuedong Zhang (zxued@sina.com)

Version: 1 Date: 28 Dec 2019

Author’s response to reviews:

Replies to the comments of the reviewer(s)

Ajay Donepudi (Reviewer 1):

1. Images of HMGB1 staining do not represent quantification results or explanations provided in the manuscript. The whole premise or hypothesis of the manuscript depends on this observation.

The reviewer's comments are very pertinent, we appreciate it very much!

Unfortunately, we only used Semiquantitative analyses including west-blot and immunohistochemistry to detect the protein expression of HMGB-1, but not quantitative method.

We thought that only the results of immunohistochemistry could be convincing.

In the experiment, we actually measured the protein expression level of HMGB-1 by the western blot method, so we also add the results of western blot detection of HMGB-1 to the article, which is located in the third paragraph of Results section and Figure 2.

Thanks very much for your kind reminder!

2. Cyclosporine A is known to inhibit Calcineurin and thereby inhibiting NFAT and NF-kB transcriptional activity resulting in decreased inflammation. HMGB-1 is also known to upregulate pro-inflammatory cytokine by activating the NF-kB pathway. Authors did not see whether in their model there cyclosporin A treatment altered Calcineurin and NF-kB pathways.

Whether or not what the reviewer express is that cyclosporin A treatment altered Calcineurin and NF-kB pathways, directly decreasing inflammation, instead of indirectly decreasing
inflammation by suppressing HGMB-1 which downregulating pro-inflammatory cytokine by activating the NF-kB pathway.

We understand the reviewer's concerns!

Studies indicated that HMGB-1 could act as a proinflammatory cytokine and participate in various diabetic complications including retinopathy, cardiomyopathy and nephropathy [1-3]. HMGB-1 was reported to be involved in the pathogenesis of diabetic retinopathy activation of inflammatory cascades [4]. Furthermore, retinal upregulation of HMGB-1 was demonstrated to participate in retinopathy by increasing blood-retinal barrier permeability in the rat model of streptozotocin (STZ)-induced DM, possibly through the production of various proinflammatory cytokines [5].

Although as you said “Cyclosporine A is known to inhibit Calcineurin and thereby inhibiting NFAT and NF-kB transcriptional activity resulting in decreased inflammation. HMGB-1 is also known to upregulate pro-inflammatory cytokine by activating the NF-kB pathway”, our topic has been focused on the role of cyclosporin A and HMGB-1 in high glucose environment. In addition, Miriam BF et al found that cyclosporin A inhibits colon cancer cell growth independently of the calcineurin pathway [6].

So, we did not did not see whether in our model there cyclosporin A treatment altered Calcineurin and NF-kB pathways.


3. The authors should provide retinal pro-inflammatory gene expression in cyclosporine A treated diabetic rats.
Your good advice was very much appreciated!

We provided retinal pro-inflammatory gene expression in cyclosporine A treated diabetic rats, and added the experimental data to the article in the fourth paragraph of Results section and figure 3.

4. The authors should show both pro-IL-1β and mature IL-1β expression in the western blot analysis.

Hyperglycemia has been implicated as an important contributing factor in DR progression through damaging the retinal microvasculature, resulting in retinal structure and function disorder [7]. In 2002, the group of Prof. Tschopp described a multiprotein complex able to oligomerize and activate inflammatory caspases leading to the processing of IL-1β and IL-18. This complex was named NLR PYD-containing protein 3 (NLRP3) inflammasome [8]. In an excellent agreement, the present data revealed that hyperglycemia could induce the activation of the NLRP3 inflammasome and ultimately lead to the increased expression of IL-1β and IL-18. IL-1β is an inducible cytokine and is not generally expressed in healthy cells or tissue [9].

In our previous research, we have found that compared with the low-glucose culture, high glucose triggered higher cell death and increased IL-1β mRNA expression and protein production in retinal pigment epithelial cell [10].

In the current study, we believe that mature IL-1β plays an obvious role in promoting inflammation in high glucose environment. Therefore, the purpose of the experiment is that cyclosporine A inhibits the formation of mature IL-1β in high glucose environment, thus plays an anti-inflammatory role.

Therefore, we only measured the important mature IL-1β, but not the pro-IL-1β in the experiment.

We are deeply sorry for such lack of detection in pro-IL-1β.

Thanks very much for your comments and suggestions on our article.

We really appreciate your work for the review!

7, Shen Hongjie, Rong Hua, Pterostilbene impact on retinal endothelial cells under high glucose environment. Int J Clin Exp Pathol, 2015, 8: 12589-12594.


Thanks very much for your comments and suggestions on our article.

We really appreciate your work for the review!

Lola Mugisho (Reviewer 2)

1, The experiment comprised of 2 parts. In the first part, the effect of cyclosporin A was evaluated in normal and diabetic patients. In the second part, only normal, healthy rats were subjected to treatment. This is confusing because the authors aimed to evaluate the effect of HGMB1 activation in diabetic not normal rats. Could the authors explain the rationale for this experimental design?

The reviewer's comments are very pertinent, we appreciate it very much!

In the first part, we evaluated the effect of cyclosporin A in normal and diabetic patients. In the second part, only normal, healthy rats were subjected to treatment, why?

In previous studies, Mohammad et al [1] showed that intravitreal administration of HMGB-1 protein activates inflammatory signaling pathway components and disrupts retinal...

2. For the second part of the study where HMGB-1 was injected intravitreally, do the authors have any proof that the HMGB-1 was taken up by the retina? Is the expression profile of HMGB-1 discussed in figure 2 likely to be similar to the uptake?

This is a rigorous and tough question, it reflects the reviewer's preciseness in scientific research, we appreciate it very much!

In previous studies, Mohammad et al showed that intravitreal administration of HMGB-1 to normal rats caused HMGB-1 expression increased in the retinas by about 80% compared to the values obtained from the contralateral eye that received PBS alone. So, we can think that the HMGB-1 was taken up by the retina in the second part of the study. In view of this, our study has not repeated such a test.

HMGB-1 protein is a multifunctional protein, which is mainly present in the nucleus where it stabilizes nucleosome formation and facilitates transcription. Extracellular HMGB-1 has been recognized as a pro-inflammatory mediator and more recently, as a proangiogenic factor [2,3]. Necrotic cell death can result in passive leakage of HMGB-1 from the cell as the protein is then no longer bound to DNA inducing inflammatory response and promoting tissue repair and angiogenesis [4].

Based on the above, we reasoned that hyperglycemia leads to the necrosis of retinal endothelial cells and RPE cells, thus releasing HMGB-1, which promotes the aggravation of retinal inflammation. When HMGB-1 was injected into vitreous cavity, it should be an extracellular substance, which caused “diabetic-like” retinopathy, and can promote inflammation of retina, lead to the necrosis of retinal endothelial cells, and release HMGB-1 which in cells.

So, we assumed that the expression profile of HMGB-1 discussed in figure 2C and 2D, not 2A and 2B, likely to be similar to the uptake. Because figure 2A and 2B are not the retina of a diabetic model.

The reviewer's comments give us a very interesting and challenging research point.

In the follow-up study, we will study this issue separately, expecting to give the reviewer further explanation.


3, DMSO was used for control in the first part of the study. However, DMSO is a well-known cytotoxic agent. Were there any toxic effects of DMSO observed during the study? What concentration of DMSO was used?

Systemic application of CsA has hepatotoxicity and nephrotoxicity, and due to the existence of blood retinal barrier, it is difficult to achieve the ideal concentration in the eyes [5]. When the concentration of CsA is 100-1000 \( \mu \text{g/L} \), it can play a role in regulating the immunosuppression of inflammation [6]. When CsA concentration in vitreous cavity is 10-1000 \( \mu \text{g/L} \), it has no toxic effect on rhesus monkey retina [7]. CsA is a water-insoluble drug, and its solvent DMSO is both lipid-soluble and water-soluble. It has been shown that DMSO can be injected into the vitreous cavity as a solvent [8,9]. When the final concentration of DMSO in vitreous cavity is less than or equal to 0.1% (2.96\( \times 10^{-5} \) \( \mu \text{M} \)), it has no toxic effect on the retina [10].

So, the concentration used in our experiment is 0.1% (2.96\( \times 10^{-5} \) \( \mu \text{M} \)), and we didn't observe any toxic effects of DMSO during the study.


In Figure 1, the authors described the presence of oedema within the GCL layer in the diabetic group. Could the authors comment on whether this is the usual pathology seen in DR patients and how reliable it is to assume the presence of oedema from H&E images?

The blood-retinal barrier (BRB) is a tissue structure with selective permeability between retinal ganglion cell layer and blood [11]. The BRB plays a fundamental role in the structural and functional integrity of the retina, regulating the flux of molecules within the eye and protecting the retinal tissue from pathogens. [12,13] Breakdown of the BRB can thus occur either at the level of retinal vessels or at the RGC layer [14]. Upregulation of cytokines and other proinflammatory mediators leading to persistent low-grade inflammation is believed to actively contribute to the DR-associated damage to the retinal vasculature, inducing breakdown of the BRB, subsequent retinal edema formation [15]. So, the presence of oedema within the RGC layer is the usual pathology seen in DR patients.

Diabetic retinopathy (DR) is one of the most common microvascular diseases in diabetes [16]. In order to better explore the molecular and cellular pathogenesis of DR, researchers have conducted extensive research on animal models of diabetes [17,18]. Among them, streptozotocin-induced diabetic retinopathy in mice is a very typical and classic animal model. It shows rapid hyperglycemia (3 days after injection) and some pathological changes of early diabetes, such as loss of peripheral cells and capillaries, thickening of vascular basement membrane, closure of blood vessels and retinal edema formation. And the HE staining is the classical method to evaluate the retinal edema of model mice [19]. We think it is reliable to evaluate the existence of retinal edema by HE images.


Other comments

ABSTRACT

* Methods - line 52/53 "Another part of normal wild-type rats was subjected to…”

♣ Reword - Another group of normal wild-type rats were subjected to?

We have made the amendments in the Abstract section, line 52/53, page 2("Another part of normal wild-type rats was subjected to” change to “Another group of normal wild-type rats were subjected to”)

BACKGROUND

* Line 41 - parentheses are needed for IL-1b & TNF-a section, eg "…inflammatory cytokines, namely interleukin-1B… (TNF-a), are elevated in…”

* Line 49/50 - reword "recent researches" - researches is not grammatically correct

o "Recent research” or "Recent studies"

We have made the amendments in the Background section, line 41, line 49/50(“Recent researches” change to “Recent studies”) page 3

METHODS
Intravitreal treatment of Cs-A/HMGB-1

- Line 26/27 - Reword - "Two parts of experiments were designed in this study." to say "This study comprised of two experimental parts"?
  - Line 49 - "none-diabetic" change to "non-diabetic"

Immunohistochemistry & Western Blot

- Line 32 - "(Abcam)" - need more of the specifications etc, eg dilution, catalog number, company address

We have made the amendments in the Methods section, paragraph 3, line 26/27 ("Two parts of experiments were designed in this study." Change to "This study comprised of two experimental parts"), line 49 ("none-diabetic" change to "non-diabetic")

We also have made the amendments in the Methods section, paragraph 5, line 32 (1:500, Abcam, Cambridge, UK).

RESULTS

- Animal characteristics
  - Line 54/55 - "P<0.01" - p-values for significant should all be lower case & italics
    - Ie "p < 0.01" - repeats throughout paper/figure legends

- Pathomorphological alternations of retinal tissues is not grammatically correct. Please rephrase appropriately

- Line 4 - "HE staining" - please rephrase.

We have made the amendments in the Results section and in Figure legends ("P<0.01" change to "p<0.01").

We also have made the amendments in the Results section, paragraph 2, line 1 ("Pathomorphological alternations of retinal tissues" change to "The pathomorphological changes of retinal tissues"), line 2 ("HE staining" change to "Retinal tissues with HE staining"

DISCUSSION

- Line 6 - reword - "proved" - eg showed
* Line 6 - not sure that you can say that it had "an inhibitory effect on diabetes-caused retinopathy" as retinopathy was not measured directly in this study. Please rephrase appropriately.

* Line 6/8 - reword - "Similarly, inhibitory effect of Cs-A was showed in vitro". Change to "Similarly, an inhibitory effect of Cs-A was shown in vitro"

We have made the amendments in the Discussion section, line 6 ("In the present study, we proved that Cs-A has an inhibitory effect on diabetes-caused retinopathy" change to “In the present study, we showed that Cs-A could attenuate retinal edema in diabetes-caused retinopathy”), line 6/8 ("Similarly, inhibitory effect of Cs-A was showed in vitro". Change to "Similarly, an inhibitory effect of Cs-A was shown in vitro")

FIGURES

* Figure 1

  o Require comments to explain the larger ONL seen in C & D versus A & B

    o The authors suggest that treatment seen in figure 1D is better than in figure 1C, however this is unclear from the images. Is there any quantification to support this statement?

The HE staining is the classical method to evaluate the retinal edema of model mice. From the images, we could find the differences clearly and intuitively. This is a kind of qualitative, not a kind of quantification. This kind of qualitative analysis has been used in previous studies [20]. For the differences in images, we made a detailed analysis and explanation in Results section and in figure 1 legend.


* Figure 2

  o Figure - symbol chosen to indicate significance for DM comparison is not clear. Could the authors consider the use of "+" instead?

    o Representative images in figure 2A-D do not match the quantification data presented in figure 2E.

We didn't choose "+" as a symbol mark, instead we considered the use of “*” and “※” as a symbol mark.
We thought that the results of immunohistochemistry and western blot could be convincing. In the experiment, we actually measured the protein expression level of HMGB-1 by the western blot method, so we also add the results of western blot detection of HMGB-1 to the article, which is located in the third paragraph of Results section and Figure 2. In addition, some errors have been made in the selection of pictures (Figure 3C change to Figure 3D), we made the amendments in the Figure 2.

* Figure 3

  o As the column bars have been differentiated through shading, it would be better to add a legend and remove the treatments names from the x-axis. This is consistent for all similar figures.

  o The y-axis is presented as "pg/mg" however this is not adequately described in the figure legends. Therefore, is this in pg per mg of retina or the entire eyeball, for instance?

We removed the group names from the x-axis in all similar figures.

“pg/mg” in the y-axis means pg per mg of retina, not the entire eyeball.

We described in the figure 3 legends.

* Figure 4

  o the methods suggest that the western blot analysis was carried out in comparison to the actin antibody. However, the figure suggests that the housekeeping gene used was GAPDH. Could the authors please clarify?

After a thorough examination of the entire article, we found that the comparison to the actin antibody in the methods was a clerical error. We revised it in the article and in Figure 4.

We are deeply sorry for such clerical error.

Thanks very much for your comments and suggestions on our article.

We really appreciate your work for the review!