

Biphasic Protective Effects of Macrophage Migration Inhibitory Factor in Ischemia/Reperfusion-Induced Acute Kidney Injury

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The paper identified the protective effects of MIF in the I/R model of AKI and attempted to investigate the underlying mechanisms. Although the topic of this study is interesting, I still have several comments regarding this study:

1. This is an interesting study. However, I found that several previous publications have already reported the renoprotective effects of MIF in acute kidney injury, so this study lacks novelty. For example, Stoppe C, et al. found that MIF could restore ferroptosis in I/R-induced AKI and attenuate injury using genetic knockout mice (doi:10.1126/scitranslmed.aan4886).
2. The authors proposed that MIF activates AMPK and further increases GPX4. However, the data did not support this view. It was previously reported that there was no difference in AMPK activation by acute ischemia in MIF knockout and wild-type mice. (doi:10.1371/journal.pone.0029887) I suggest the authors add experiments (such as using inhibitors of AMPK signaling and MIF siRNA) to verify that MIF protects AKI via AMPK signaling.
3. The quality of the WB may need further improvement (e.g. Figure 3H).

Reviewer #3

(Remarks to the Author)

In this manuscript, Du et al explored the biphasic protective effects of MIF in ischemia reperfusion induced AKI. They found that MIF was upregulated in both the ischemia stage and in the reperfusion stage in vivo and during hypoxia and reoxygen in vitro. Upregulation and downregulation of MIF could change ferroptosis indicators, indicating MIF would regulate the level ferroptosis during I/R. And they further found that the regulation of ferroptosis by MIF was independent of GPX4 activity and AMPK phosphorylation during reperfusion stage, but was quite associated with GPX4 activity and AMPK phosphorylation during ischemia stage. Overall, this study was well designed and the results were convincing. Some attention should be paid to the result explanation. And the questions were listed as below:

1. Is Figure 2 A a duplicate of Supplementary Figure 2 A and B?
2. Supplementary Figure 3, What is the method of administration for Z-vad, Nec-1 and Fer-1? The Flow diagram says i.p, but the text says "via the tail vein".
3. The interpretation of the results of Figure 2P, "suggesting that a large amount of MIF may be released from damaged renal tubular cells into plasma", seems to be overstated. The present results only show that circulating MIF increases after kidney injury, but can not indicate that circulating MIF is released from renal tubular epithelial cells.
4. Whether Figure3 implies that elevated MIF inhibits ferroptosis by increasing GSH levels but not activating GPX4 activity and the AMPK pathway, the authors should have interpreted the results in full in the last sentence.
5. Please re-summarize the major findings of this research to reflect the biphasic protective role of MIF on ferroptosis during ischemia/reperfusion kidney injury in the first paragraph of the Discussion
6. The function of MIF on anti-oxidation during reperfusion satage represented by the upregulation of GSH but not affecting the GPX4 and AMPK pathway should be discussed.
7. Did the authors detect the plasma level of MIF in only the ischemia model? The regulation of circulation level of MIF was less studied in this research and could be discussed in limited sentences.
8. In the last paragraph, this research can not suggest MIF as a biomarker for AKI.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The author has already answered my questions and doubts.

Reviewer #3

(Remarks to the Author)

In this revised manuscript, the authors have satisfactorily answered my questions and concerns. The data quality has been much improved. And the discussions are more clear. I have no further questions.

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Response

Thanks to the editors and reviewers for their hard work and constructive comments. We are sorry for the long delay in submitting the fixed version. Because the first author is currently studying for a doctor in a different school, the doctor's task is heavy, and he cannot take good care of the revision task of this article, resulting in a relatively long time. We are very grateful to the editor for giving us sufficient understanding and time to revise the manuscript. According to the suggestions put forward by the reviewers, we have made replies one by one after reviewing the literature and supplementing the experiments.

Reviewers' comments:

Reviewer #2 (Remarks to the Author):

The paper identified the protective effects of MIF in the I/R model of AKI and attempted to investigate the underlying mechanisms. Although the topic of this study is interesting, I still have several comments regarding this study:

- 1. This is an interesting study. However, I found that several previous publications have already reported the renoprotective effects of MIF in acute kidney injury, so this study lacks novelty. For example, Stoppe C, et al. found that MIF could restore ferroptosis in I/R-induced AKI and attenuate injury using genetic knockout mice (doi:10.1126/scitranslmed.aan4886).**

Response: Thanks for your suggestive advice. First of all, we must admit that we also read this article during our research, which is of great reference significance to our research, and we also compare and discuss the content and experimental results of this article in the discussion part. Second, we do not believe that even reporting the same effect of the same molecule in the same disease makes the article less innovative. Since the two articles are different in terms of mechanism, we believe that the revised article is more inclined to the macroscopic protective effect on the whole, focusing on the observation of blood biochemical indicators, pathological manifestations and inflammatory indicators, while our study is generally inclined to the specific mechanism of MIF inhibiting iron death in different periods of AKI. Therefore, from this point of view, our research can be said to be more focused on a specific direction and mechanism exploration than this research. Third, our study explored for the first time the indexes of iron death in vivo and in vitro in the kidney during the period of simple ischemia, as well as the dynamic changes of MIF during this period, which have not been involved in previous studies, so we believe that this study still has some innovative points. The innovation of this paper and the comparison and differences with previous studies are discussed in the discussion section.

- 2. The authors proposed that MIF activates AMPK and further increases GPX4. However, the data did not support this view. It was previously reported that there was no difference in AMPK activation by acute ischemia in MIF knockout and wild-type mice. (doi:10.1371/journal.pone.0029887) I suggest the authors add experiments (such as using inhibitors of AMPK signaling and MIF siRNA) to verify that MIF protects AKI via**

AMPK signaling.

Response: Thanks for your constructive advice. First, we read this literature and we think there's a big difference in the models that leads to the different conclusions. In this research (doi:10.1371/journal.pone.0029887), the ischemia model was induced by unilateral kidney clamp for 10 minutes, which is a relatively mild model compared to our bilateral ischemia for 10, 20 and 30 minutes. Second, the authors only compared AMPK phosphorylation levels in the ischemic and non-ischemic kidneys in the WT and MIF^{-/-} groups, and did not compare AMPK phosphorylation levels in the ischemic WT and MIF^{-/-} kidneys. Moreover, there was no quantitative analysis of WB results, which could not completely determine the level or activity change of AMPK phosphorylation in renal ischemia after MIF knockout. Finally, we adopted the reviewer's suggestion to increase the cell experiment using AMPK inhibitor CC during hypoxia, and the experimental results showed that the addition of CC inhibited AMPK phosphorylation and GPX4 expression. The results were shown in Supplementary Figure 8. Therefore, in summary, we believe that our results can indicate that MIF can regulate ferroptosis by activating AMPK and then up-regulating GPX4 during renal ischemia.

3. The quality of the WB may need further improvement (e.g. Figure 3H).

Response: Thanks for your suggestions. We've tried our best to improve the quality of WB brands and replaced them. Please refer to it in the revised figure legends. Please refer to Figure 3 in the revised Figure legend.

Reviewer #3 (Remarks to the Author):

In this manuscript, Du et al explored the biphasic protective effects of MIF in ischemia reperfusion induced AKI. They found that MIF was upregulated in both the ischemia stage and in the reperfusion stage in vivo and during hypoxia and reoxygen in vitro. Upregulation and downregulation of MIF could change ferroptosis indicators, indicating MIF would regulate the level ferroptosis during I/R. And they further found that the regulation of ferroptosis by MIF was independent of GPX4 activity and AMPK phosphorylation during reperfusion stage, but was quite associated with GPX4 activity and AMPK phosphorylation during ischemia stage. Overall, this study was well designed and the results were convincing. Some attention should be paid to the result explanation. And the questions were listed as below:

1. Is Figure 2 A a duplicate of Supplementary Figure 2 A and B?

Response: Thanks for your kind reminder. Because there are too many pictures and data, we mixed up the pictures and did not check them carefully. The images here are indeed duplicates. We have replaced the original images in Figure 2A and Figure 2B. First, the data presented in Supplementary Figure 2 examined the changes of BUN and Cre at different time points and the

dynamic changes of ferroptosis signature proteins in bilateral I/R mouse models. Combined with these indicators, we observed that iron death damage was most severe at I/R 1d, so we then selected this time point for follow-up experiments. Therefore, in Figure 2, we only showed the detection of blood biochemical indexes, pathological changes, and ferroptosis-related indexes of I/R 1d mice.

We replaced the correct data in Pannel A-B in Figure 2 in the revised manuscript. Please refer to Figure 2.

2. Supplementary Figure 3, What is the method of administration for Z-vad, Nec-1 and Fer-1? The Flow diagram says i.p, but the text says "via the tail vein".

Response: Those three cell death inhibitors were injected intraperitoneally but not via the tail vein. I'm sorry that we made a mistake in writing the article and thanks for pointing it out. We've corrected it, and please refer to Line 283 in the revised manuscript.

3. The interpretation of the results of Figure 2P, "suggesting that a large amount of MIF may be released from damaged renal tubular cells into plasma", seems to be overstated. The present results only show that circulating MIF increases after kidney injury, but can not indicate that circulating MIF is released from renal tubular epithelial cells.

Response: Thanks for your suggestive advice. First, we draw the previous conclusion because there was literatures reporting that tubular epithelial cells are a major source of MIF in response to renal injury (PMID: 11126199, 12631343). Second, Li et al (PMID: 30077612) and Djudaj et al (PMID: 28801314) also demonstrated MIF is highly expressed and released by damaged TEC in other AKI models by using gene knockout mice and IF or IHC staining. Combining your suggestion and our current research data, we revised the statements to make them more accurate and rigorous. Please refer to Line 312-314.

4. Whether Figure 3 implies that elevated MIF inhibits ferroptosis by increasing GSH levels but not activating GPX4 activity and the AMPK pathway, the authors should have interpreted the results in full in the last sentence.

Response: Thanks for your constructive comments. In the revised manuscript, we've redescribed the results in their entirety. According to our results, elevated MIF expression ameliorated ferroptosis by increasing GSH content, without increasing GPX4 activity and activating the AMPK pathway. Please refer to Line 349-350, which are in red font.

5. Please re-summarize the major findings of this research to reflect the biphasic protective role of MIF on ferroptosis during ischemia/reperfusion kidney injury in the first paragraph of the Discussion

Response: Thanks for your constructive comments. On the basis of revising the previous questions, we reviewed the results of the paper and systematically and rigorously described how

MIF plays a protective role in ischemia and reperfusion stages, respectively. While describing the different mechanisms of action in the two periods, we also emphasized the innovation points of this paper. Secondly, we also described the circulatory level results of MIF in the two periods. Due to blood vessel occlusion during ischemia and no significant changes in circulation level, blood vessels are no longer being camped during reperfusion, at which time the MIF circulation level is significantly increased, suggesting that these elevated MIF are likely to come from injured kidneys. Combined with multiple previous literature reports, we believe that this conjecture is reasonable. Please refer to Line 433-449.

6. The function of MIF on anti-oxidation during reperfusion stage represented by the upregulation of GSH but not affecting the GPX4 and AMPK pathway should be discussed.

First of all, there is no clear report in the existing literature that MIF can activate AMPK signaling pathway in the reperfusion stage, and there is only a report that MIF can protect myocardial function by activating AMPK signaling in ischemic myocardium (PMID: 18235500). Therefore, from the current literature evidence, although our experimental organs are not the same as those reported in previous studies, the results are similar, so we think it is reasonable not to increase GPX4 expression by activating AMPK signaling during renal reperfusion. Secondly, MIF has previously been reported to have the effect of reducing oxidative stress (PMID : 20831446,29908183). It can be seen that MIF has the protective effect of up-regulating GSH content and anti-oxidative stress, which is also confirmed by our experimental results. Finally, GSH plays an important role in the signaling pathway of ferroptosis. Our results suggest that upregulation of GSH by MIF during reperfusion can alleviate ferroptosis. Of course, we should acknowledge the shortcomings of this study, and perhaps more attention should be paid to the effects of MIF on oxidative stress pathways during reperfusion, rather than focusing on AMPK signaling. For this part, we have added a description in the discussion part, and compared our experimental results with previous research reports, and made a discussion summary. Please refer to Line 475-483.

7. Did the authors detect the plasma level of MIF in only the ischemia model? The regulation of circulation level of MIF was less studied in this research and could be discussed in limited sentences.

Response: Thanks for your constructive comments. According to your suggestion, we examined the MIF circulation level during ischemia when we supplemented the experiment. The results was added in Supplementary Figure 9. The experimental results suggested that the mean MIF circulating water in different ischemic periods did not change significantly compared with the sham group. Therefore, based on our current results, we preliminarily suggest that neither kidney releases renal source MIF into the peripheral circulation in the case of vascular occlusion. Please refer to Line 488-494.

8. In the last paragraph, this research can not suggest MIF as a biomarker for AKI.

To be used as a biomarker, a molecule must at least meet the following characteristics: specificity, sensitivity, repeatability and stability, clinical relevance, and detectability. The results of this study indicate that MIF can be detected in AKI renal tissue and peripheral circulation level, but whether it has sensitivity and specificity needs to be expanded sample size and studied in clinical disease cohort. Therefore, the conclusion that MIF can be used as a biological marker of AKI based solely on this study is not rigorous. In response to comments from the reviewers, we have revised this sentence and believe that the statement "MIF is a potential target for the treatment of AKI" is more appropriate. Please refer to Line 521-529.