

Targeting ALOX5 ameliorates renal tubular injury in ischemia reperfusion induced acute kidney injury via ferroptosis

Corresponding Author: Dr Yunlin Feng

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The study titled "Targeting ALOX5 to inhibit ferroptosis ameliorates renal tubular injury in ischemia-reperfusion induced acute kidney injury" This study presents a significant contribution to the field of acute kidney injury (AKI) by identifying ALOX5 as a key mediator of ferroptosis in renal tubular cells during ischemia-reperfusion injury (IRI). The authors demonstrate that ALOX5-driven lipid peroxidation exacerbates tubular injury and that pharmacological inhibition with Benzbromarone (BBR) mitigates ferroptosis and improves renal outcomes. The manuscript provides some valuable insights for the future research directions to address the challenges posed by ischemia-reperfusion injury. However, major modifications are needed before potential publication:

1. Additional validation of BBR-ALOX5 specificity in vivo (e.g., off-target effects, dose-response data) would strengthen the therapeutic claim;
2. while the link between ALOX5 and ferroptosis is well-supported, the potential interplay with other cell death pathways (e.g., apoptosis, necroptosis) in IRI-AKI warrants discussion.
3. P3, Why ALOX5 was prioritized for investigation over other ferroptosis regulators (e.g., GPX4, system Xc⁻), authors should state the rationale for focusing on ALOX5.
4. P4, line 109, Due to the version or the date the data was downloaded from CTD is crucial for ensuring the reproducibility of the target identification step in the network pharmacology analysis. Please provide the version and/or access date for the CTD database.
5. P5, The statement "until the fourth generation" is ambiguous, does the experiments conducted at the fourth generation or including the four generation? Due to cell behavior can vary significantly beyond early passages. Specify the exact passage number(s) during which H/R modeling and BBR treatment were performed to ensure reproducibility.
6. P6, The term "surrounding tumor kidney tissue" is ambiguous—does this refer to histologically normal parenchyma adjacent to the tumor, or does it include peri-tumoral stroma that may exhibit tumor-associated changes? Using tissue near renal cell carcinoma as "control" requires explicit confirmation that these samples show no pathological alterations when compared to true healthy kidney tissue.
7. P6, line 173, Cervical dislocation in conscious mice 24 hours post-surgery raises significant welfare and methodological concerns, due to AVMA guidelines classify cervical dislocation in conscious rodents as acceptable only when animals are anesthetized or when immediate loss of consciousness is verified. Performing this on conscious mice recovering from major surgery likely causes severe distress and pain. Additionally, Stress from unanesthetized euthanasia may alter biomarkers (e.g., serum creatinine/BUN) and tissue integrity, compromising downstream analyses.
8. P7, The stock solution contains 5% DMSO (v/v), but the final concentration administered to mice is not specified after dilution to "needed concentrations", So, author should clarify the DMSO concentration in injected solutions.
9. P10, line 258, p should be in italic, and add one blank before and after <, including the whole manuscript.
10. P11, line 308, authors state "after IRI treatment were significantly reduced 309 in the Alox5 ^{-/-} mice in comparison to those in the wild-type mice", should provide the level of p value.
11. P12, line 324, about "significant" decreases, authors should provide the quantitative statistic analysis.
12. P 13, line 345, is BBR a bona fide ALOX5 inhibitor, please explain it from the biological relevance.
13. P17, line 463, please rephrase "This study reported for the first time that ALOX5 is up-regulated in the renal tubular cells.....", avoids overclaiming "first" while emphasizing contribution.
14. P19, Line 493, please unify the style of all the references.

Reviewer #3

(Remarks to the Author)

In this manuscript, the authors investigated the role of ALOX5 in ischemia-reperfusion kidney injury, and they reported that targeting ALOX5 might improve tubular damage in renal IRI, and the improvement is linked to ferroptosis. Overall, it is interesting for the renal diseases field. I think further improvement will certainly help to polish the reporting before publication.

1. The contents in some subsections of the Methods are too detailed, while those in the other subsections are very brief. Please keep a consistent tone throughout and move some contents to supplementary as necessary.
2. The underlying mechanism of ALOX5 in mediating tubular damage in renal IRI has not been completely clarified in this study. The findings of this study do show that ferroptosis is involved in this process, evidenced by the morphological changes in mitochondria under EM and changes in ferroptosis markers; however, the details are still not clarified. The authors should mention this limitation in the discussion.
- 3.. Please add more discussion about whether these findings can be translated into other AKI models.
4. It would be better if the authors give a deeper discussion on the clinical implications of the findings.
5. Figure 1D: The labels for "Sham I/R" and "HE PAS" appear to be reversed. Please verify and correct as needed.
6. Figure 2B: Is the ATN (acute tubular necrosis) image stained with PAS or HE? This should be clarified in the figure legend or methods.
7. In Figure 6B, the creatinine level for WT I/R is reported as $\sim 100 \mu\text{mol/L}$. However, in Figure 3B, the same condition shows a level of $\sim 20 \mu\text{mol/L}$. Please check the data.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised manuscript demonstrates significant progress in response to the initial reviewer concerns. The authors have substantially improved the manuscript's scientific rigor, methodological transparency, data interpretation, and clarity of language.

However, regarding the Figure 4, the authors describe that the inhibited HK-2 cell proliferation upon the BBR treatment, starting from $32 \mu\text{M}$ to an IC_{50} of $41.89 \mu\text{M}$, so we can find that the therapeutic window is very narrow, and it's a bit hard to understand. Based on common sense, additionally, how to understand the $R^2=1$ in Figure 4C, which is also not conform to common sense. We think the author should carefully check the raw data, or repeat the work again.

Reviewer #3

(Remarks to the Author)

I have no other comments on the revised manuscript

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the revised version of the manuscript, the authors have responded to the suggestions made by the reviewer. In my opinion, the manuscript may be accepted for publication.

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Dear Editor and Reviewers,

Thank you very much for the valuable comments that help us to improve our work and the opportunity to revise our manuscript. On behalf of all authors, I am submitting the revised manuscript with changes highlighted in yellow for your further assessment. Please find our point-to-point response below to address the editor and reviewers' concerns. A clean version of the final manuscript is also submitted as a supplementary document for your reference. We hope that you find our responses and modifications satisfactory.

Please note we have revised the title to "Targeting ALOX5 ameliorates renal tubular injury in ischemia-reperfusion induced acute kidney injury via inhibition of ferroptosis" to make it more readable. The title in the system has been revised as well.

Reviewer #1 (Remarks to the Author):

The study titled "Targeting ALOX5 to inhibit ferroptosis ameliorates renal tubular injury in ischemia-reperfusion induced acute kidney injury" This study presents a significant contribution to the field of acute kidney injury (AKI) by identifying ALOX5 as a key mediator of ferroptosis in renal tubular cells during ischemia-reperfusion injury (IRI). The authors demonstrate that ALOX5-driven lipid peroxidation exacerbates tubular injury and that pharmacological inhibition with Benzbromarone (BBR) mitigates ferroptosis and improves renal outcomes. The manuscript provides some valuable insights for the future research directions to address the challenges posed by ischemia-reperfusion injury. However, major modifications are needed before potential publication:

1. Additional validation of BBR-ALOX5 specificity in vivo (e.g., off-target effects, dose-response data) would strengthen the therapeutic claim;

Thank you very much for this comment. We acknowledge that additional validation of the specificity is quite necessary for the therapeutic claim. The molecular interaction experiments using Octet RED96e system indicated that the interaction between BBR and the ALOX5 recombinant protein exhibited a concentration-dependent manner, with a binding KD of 5.28×10^{-5} (Fig 5. B & C). However, at this stage in this work, the target binding sites between ALOX5 and BBR were derived through molecular simulation and have not been validated by bench experiments, for example, site-specific knockout methods, which will be our next step of work. In addition, it is possible that BBR might interact with other targets, therefore bringing up off-target effects in real application. Although this is not explored in this study, we should have discussed this due to the concern of drug safety. To address your concern, we have now added content on this point in the Discussion, the 5th paragraph.

2. while the link between ALOX5 and ferroptosis is well-supported, the potential interplay with other cell death pathways (e.g., apoptosis, necroptosis) in IRI-AKI warrants discussion.

Thank you for this suggestion. Regulated cell deaths mechanisms play a critical role in renal tubular cell injury associated with AKI, including process such as apoptosis, pyroptosis, ferroptosis, and autophagy. In this piece of research, we mainly focused on ferroptosis, and we acknowledge that the regulation of ferroptosis is a complex network and ALOX5 might be one among the multiple involving pathways. In addition, the interplay between ALOX5 and other cell death pathways is possible, since ALOX5 mediates lipid peroxidation, which might relate to other cell death pathways via disturbance in energy metabolism and oxidative stress. We have revised the Discussion and added this point. Please see the revised Discussion, the 3rd paragraph.

3. P3, Why ALOX5 was prioritized for investigation over other ferroptosis regulators (e.g., GPX4, system Xc⁻), authors should state the rationale for focusing on ALOX5.

Thank you for this comment. The main reason for choosing ALOX5 as the research subject is a result of our long-term investigation in the AKI area. The main theme of our research team is molecular mechanism of tubular injury

in AKI. Accumulating evidences have confirmed that ferroptosis is important in tubular injury in AKI, and it is reported that ALOX5 metabolizes arachidonic acid to produce toxic 5-hydroxyeicosatetraenoic acid (5-HETE), leading to ferroptosis [1, 2]. Therefore, ALOX5's role of regulating fatty acid metabolism leads us to investigate that whether ALOX5 is involved in renal tubular injury during AKI via the ferroptosis pathway. And we acknowledge that our research starts with clues derived from bioinformatic analysis of proteomic data. To make the picture complete, we organized the “-omic” data in the Results, not the Introduction.

4.P4, line 109, Due to the version or the date the data was downloaded from CTD is crucial for ensuring the reproducibility of the target identification step in the network pharmacology analysis. Please provide the version and/or access date for the CTD database.

The CTD database was accessed on 1 December, 2022. We have added this information in the Methods. Please see the revised contents in *Materials and Methods/Bioinformatics analysis*.

5.P5, The statement "until the fourth generation" is ambiguous, does the experiments conducted at the fourth generation or including the four generation? Due to cell behavior can vary significantly beyond early passages. Specify the exact passage number(s) during which H/R modeling and BBR treatment were performed to ensure reproducibility.

The HK-2 cells used in this study were purchased from the cell bank of the Typical Culture Preservation Committee of the Chinese Academy of Sciences, and were cell lines rather than primary cells. The cells were cultured according to the instructions of manufactures. Human renal epithelial cell line HK-2 cells contain the recombinant retroviral vector pLXSN 16 E6/E7 carrying the HPV-16 E7/E7 gene, and retain the functional characteristics of proximal renal tubular epithelial cells [3]. We have provided the information in the Methods. Please see the *Materials and Methods/Cell culture and treatment*.

6.P6, The term "surrounding tumor kidney tissue" is ambiguous—does this refer to histologically normal parenchyma adjacent to the tumor, or does it include peri-tumoral stroma that may exhibit tumor-associated changes? Using tissue near renal cell carcinoma as "control" requires explicit confirmation that these samples show no pathological alterations when compared to true healthy kidney tissue.

Thank you for this comment. We acknowledge the term of “surrounding tumor” might be misleading. Therefore, we have revised the term to “adjacent non-tumorous kidney tissue” to make the statement more accurate. Figure 2 and its legends have been revised accordingly. Please see the corresponding contents.

7.P6, line 173, Cervical dislocation in conscious mice 24 hours post-surgery raises significant welfare and methodological concerns, due to AVMA guidelines classify cervical dislocation in conscious rodents as acceptable only when animals are anesthetized or when immediate loss of consciousness is verified. Performing this on conscious mice recovering from major surgery likely causes severe distress and pain. Additionally, Stress from unanesthetized euthanasia may alter biomarkers (e.g., serum creatinine/BUN) and tissue integrity, compromising downstream analyses.

All mice had been anesthetized by intraperitoneal injection of 1% sodium pentobarbital solution (50 mg/kg) before being euthanized using the cervical dislocation method. We have revised the statement to make it more precise. Please see the revised contents in *Materials and Methods/ Animals and ischemia/reperfusion (I/R) modelling*.

8.P7, The stock solution contains 5% DMSO (v/v), but the final concentration administered to mice is not specified after dilution to "needed concentrations", So, author should clarify the DMSO concentration in injected solutions.

The “needed concentrations” here refers BBR. The stock solution contains 5% DMSO (v/v). The DMSO concentration in the final solutions administrated to mice was 0.1% DMSO (v/v). We have added this information in the main contents accordingly. Please see the revised *Materials and Methods/Animal BBR treatment*.

9.P10, line258, p should be in italic, and add one blank before and after <, including the whole manuscript.

Thank you for pointing out this mistake. We have made necessary corrections as mentioned throughout the manuscript.

10.P11, line 308, authors state "after IRI treatment were significantly reduced 309 in the Alox5 ^{-/-} mice in comparison to those in the wild-type mice", should provide the level of p value.

The significance is evidenced by p values from the semi-quantitative statistical analysis. We have provided the p values from the semi-quantitative statistical analysis in Figure 3F at the above place. Please see the revised contents.

11.P12, line 324, about "significant" decreases, authors should provide the quantitative statistic analysis.

Thank you for this comment. As mentioned above, we have provided the p values from the semi-quantitative statistical analysis in Figure 4 D-F at the above place. Please see the revised contents.

12.P 13, line 345, is BBR a bona fide ALOX5 inhibitor, please explain it from the biological relevance.

BBR is a widely used pharmacological agent for reducing serum uric acid levels. It functions by inhibiting the expression of urate transporter 1 in the renal proximal tubule, thereby decreasing the reabsorption of uric acid by the kidneys, enhancing uric acid excretion, and consequently lowering blood uric acid levels. Research has demonstrated that BBR may mitigate cisplatin-induced nephrotoxicity. It has not been reported as an ALOX5 inhibitor before. We have identified BBR through molecular screening and confirmed its protective effects in mice model. Whether BBR can be used as an effective drug awaits in-depth investigation which is beyond the scope of this work.

13.P17, line 463, please rephrase "This study reported for the first time that ALOX5 is up-regulated in the renal tubular cells.....", avoids overclaiming "first" while emphasizing contribution.

Thank you very much for helping us to be more accurate. We have rephrased this sentence. Please see the revised contents.

14.P19, Line 493, please unify the style of all the references.

We have unified the reference style using the **Nature** style for Endnote. Please see the revised References section.

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors investigated the role of ALOX5 in ischemia-reperfusion kidney injury, and they reported that targeting ALOX5 might improve tubular damage in renal IRI, and the improvement is linked to ferroptosis. Overall, it is interesting for the renal diseases field. I think further improvement will certainly help to polish the reporting before publication.

1. The contents in some subsections of the Methods are too detailed, while those in the other subsections are very brief. Please keep a consistent tone throughout and move some contents to supplementary as necessary.

We have now revised the statements of the human samples, animal models, and cell cultures, adding information required for replication. The contents are also re-organized in accordance to the sequence of results presentation. We hope the Methods section reads better at this time.

2. The underlying mechanism of ALOX5 in mediating tubular damage in renal IRI has not been completely clarified in this study. The findings of this study do show that ferroptosis is involved in this process, evidenced by the morphological changes in mitochondria under EM and changes in ferroptosis markers; however, the details are still not clarified. The authors should mention this limitation in the discussion.

Thank you very much for this comment. We acknowledge that this study serves as an initial exploration into the role of ALOX5 in renal IRI; however, it lacks comprehensive validation from a clinical standpoint. Therefore, it is premature to conclusively identify ALOX5 as a therapeutic target for AKI. Our subsequent objective is to establish a clinical cohort to investigate the potential of ALOX5 as a specific target for ameliorating renal tubular injury in AKI. We have mentioned this as a limitation in the Discussion. Please see the last paragraph of Discussion.

3.. Please add more discussion about whether these findings can be translated into other AKI models.

Thank you very much for pointing out this point. We acknowledge that this study exclusively examined the IRI-induced AKI model, as the initial bioinformatics analysis indicating ALOX5 upregulation was derived from proteomic analysis of IRI-induced AKI. Therefore, we utilized this model to investigate the role and mechanism of ALOX5 in tubular injury in AKI. Direct translation of the results presented in IRI-AKI to other AKI models is inappropriate. We are planning to validate whether ALOX5 exhibits similar effects in other AKI models, as this would contribute to a comprehensive profiling of ALOX5 in AKI and represents a future direction. We have mentioned this as a limitation in the last paragraph of Discussion.

4. It would be better if the authors give a deeper discussion on the clinical implications of the findings.

Thank you very much for this comment. The clinical implications of the findings include the potential value of ALOX5 as an interventional target and the possible therapeutic effect of BBR in IRI induced AKI. We have expanded the discussion on these implications in the contents in Discussion, paragraphs 3 to 5, as suggested. Please see the corresponding contents.

5. Figure 1D: The labels for "Sham I/R" and "HE PAS" appear to be reversed. Please verify and correct as needed.

We're sincerely sorry for this mistake and have corrected it. Please see the revised Figure 1.

6. Figure 2B: Is the ATN (acute tubular necrosis) image stained with PAS or HE? This should be clarified in the figure legend or methods.

Thank you very much for pointing out this error. We have corrected this mistake in the figure and the legends. Please see the revised Figure 2.

7. In Figure 6B, the creatinine level for WT I/R is reported as ~100 $\mu\text{mol/L}$. However, in Figure 3B, the same condition shows a level of ~20 $\mu\text{mol/L}$. Please check the data.

Thank you very much for pointing out this error. We have corrected the mistake in Figure 3B. Please see the revised Figure 3.

References

1. Friedmann Angeli, J.P., et al., *Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice*. Nat Cell Biol, 2014. **16**(12): p. 1180-91.
2. Li, K., et al., *ALOX5 inhibition protects against dopaminergic neurons undergoing ferroptosis*. Pharmacol Res, 2023. **193**: p. 106779.
3. Ryan, M.J., et al., *HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney*. Kidney Int, 1994. **45**(1): p. 48-57.

Dear Editor and Reviewers,

Thank you very much for the valuable comments that help us to improve our work and the opportunity to revise our manuscript. On behalf of all authors, I am submitting the revised manuscript and supplementary for the second round with changes highlighted in yellow for your further assessment. Please find our point-to-point response below to address the editor and reviewers' concerns. A clean version of the final manuscript is also submitted as a supplementary document for your reference. We hope that you find our responses and modifications satisfactory.

Please note we have provided the following files as required:

1. unedited gel images for each WB experiment in a Supplementary Data file entitled "unedited gel images";
2. the numerical source data for graphs and charts in a Supplementary Data file (in excel format) entitled "numerical source data for graphs";
3. the completed ARRIVE checklist.

Reviewer #1 (Remarks to the Author):

However, regarding the Figure 4, the authors describe that the inhibited HK-2 cell proliferation upon the BBR treatment, starting from 32 μ M to an IC₅₀ of 41.89 μ M, so we can find that the therapeutic window is very narrow, and It's a bit hard to understand Based on common sense, additionally, how to understand the R²=1 in Figure 4 C, which is also not conform to common sense. We think the author should carefully check the raw data, or repeat the work again.

Thank you very much for this comment. The inhibitory effect of BBR on HK-2 cells proliferation does show significance from 32 μ M and the IC₅₀ of BBR in HK-2 cells is 41.89 μ M, as shown in supplementary Figure 8. These results derived 5 replicates. However, the protective effect of BBR against H/R injury in HK-2 cells became obvious from 2 μ M, as shown in Figure 7B, and 2 μ M was the concentration used in the subsequent experiments to demonstrate the protective effect of BBR in cell experiments. Since 2 μ M is about one tenth of 32 μ M and we generally consider an effective concentration that is two orders of magnitude lower than the toxic concentration to represent a safe therapeutic window, we consider these results are indicative of a narrow therapeutic window of BBR in this AKI setting, although the *in vitro* results do not necessarily represent the effects *in vivo*. To avoid the misleading information in the original statement, we have revised the corresponding discussion in the 4th paragraph. Please see the revised contents highlighted in yellow in the 4th paragraph in Discussion.

Regarding the "R²=1" in Figure 5C, the R² value measures the goodness of fitting of steady state curve and its values were provided by the software. Thank you very much for this comment. We have double-checked the raw data and repeated the steady state analysis. The mistake has been corrected. Meanwhile, to present the results in a more standardized form, we have revised Figure 5 and its legends, as well as the corresponding contents in the Results section. Please see the revised contents highlighted in yellow in *Results/Benzbromarone interacts with ALOX5* and Figure 5.