Peer Review File

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Reviewer A

The article by Zhou et al. describes the involvement of Klotho in reducing sepsis-associated AKI through Nrf2 activation. The manuscript explores the involvement of the Klotho-mediated ferroptosis pathway which may lead to clinical interventions in the sepsis AKI field. However, the manuscript in the current form lacks details needed for the reviewer to confirm the statements reported. Please see the comments below to improve the readability of the manuscript. A number of key details are missing from the text. Especially, please provide separate enlarged images in the figures of staining. As presented, these panels are too small to see in order to verify the authors' claims.

1. In methods, the authors mentioned use of Lip-1 and Nrf2-IN-1 but did not mention what the compounds are and their function. Also, what concentration of each of this compound is used?
Reply 1: Liproxstatin-1（Lip-1）is an effective ferroptosis inhibitor.Nrf2-IN-1 is an inhibitor of nuclear factor-erythroid 2-related factor 2 (Nrf2) and the specific dosage has been reflected in the article (see Page 5, line 137-141).

2. Page 3 line 82, do you mean bacterial infection?
Reply 2: Yes.

3. Page 3 line 98, "iron death" – replace with "ferroptosis"
Reply 3: We have made some changes in the text (see Page 3, line 99).
Changes in the text: Previous studies have shown that ferroptosis is a key mechanism for sequential tubular cell death after ischemic kidney.

4. Page 5 line 158 authors mentioned MDA, GSH-Px and SOD as inflammatory markers but they are not; please edit to a correct descriptor.
Reply 4: MDA, GSH-Px, and SOD are markers of oxidative stress, we have made changes (see Page 5, line 159).
Changes in the text: The serum levels of the oxidative factor markers tumor necrosis

5. Page 5 line 162 what does "determined strictly" mean?
Reply 5: This refers to the operation of the experimental protocol in accordance with the kit instructions.

6. Page 5 line 167 please change the title to histopathology.
Reply 6: We have made changes (see Page 6, line 169).
Changes in the text: Histopathology

7. Page 6 line 179 please remove the term content from the title.
Reply 7: We have made remove (see Page 6, line 181).

Changes in the text: Measurement of cell viability and intracellular reactive oxygen species (ROS)

8. Please make sure ul together wherever mentioned.
Reply 8: We checked the articles to make sure they were together.

9. Please mention what microscope was used to acquire the images.
Reply 9: We use electron microscopy (HT7800/HT7700, Hitachi Ltd., Tokyo, Japan). Fluorescence microscope (DM3000 LED, Leica, Wetzlar, Germany). In addition, we have added to the article (see Page 6, line 174 and Page 7, line 202).
Changes in the text:2.5% glutaraldehyde fixative for HE staining and electron microscopic observation (HT7800/HT7700, Hitachi Ltd., Tokyo, Japan); observed and acquired under a fluorescence microscope (DM3000 LED, Leica, Wetzlar, Germany).

10. Page 7 line 204 please replaced “sealed" with “blocked.”
Reply 10: We have made changes (see Page 7, line 206).
Changes in the text: for 10 min and blocked in BAS for 1 h.

11. Page 7 line 227-230 edit for multiple errors.
Reply 11: We have made changes (see Page 7, line 231-232).
Changes in the text: In this study, GraphPad Prism 9 software was used to plot the experimental data and SPSS 27 software was used to analyze the data statistically.

12. Page 8 line 235 how did you exclude the mice? Especially, if they were treated beforehand, how do you know that the injury plus treatment resulted in a lower value rather than just less injury to begin with?
Reply 12: First of all, before the formal experiment, we explored the SA-AKI model through experiments and excluded non-SA-AKI mice according to the degree of creatinine increase. Second, the whole experiment was completed by two people who were the same as the pre-experiment, the items used were the same as the pre-experiment, and the mice were all purchased from the same biological company.

13. Page 8 235 remove “nonrenal injured” with “uninjured."
Reply 13: We have made some changes in the text (see Page 8, line 237).
Changes in the text: A total of 40 mice were included in this study after excluding dead and uninjured mice.

14. Page 9, line 285-6 needs editing
Reply 14: We have made some editing in the text (see Page 9, line 297-300).
Changes in the text: The above findings suggest that the mechanism of action of Klotho is to nephroprotective by inhibiting the iron apoptosis signaling pathway through the activation of Nrf2 (Figure 2I-J), which effectively reduced the iron content in the tissues (Figure 2K).

15. Page 9 line 290 How do you know the Klotho visualized is not the Klotho you supplied?

Reply 15: It's possible that I supplied the Klotho, but whether it was a supplement or not did no harm to the SA-AKI mice.

16. In results, please describe in detail Fig 1A -1D and all the images in all figures. Each figure that is reported should be explained in detail. Currently the description of the data is too brief.

Reply 16: At 24 h of CLP model preparation, the values of Scr and BUN in the CLP group were significantly increased, which were significantly different from those in the sham group, and the mean serum Scr and BUN in the CLP group could be increased to 96 umol/L and 21.3 mmol/L, respectively, and the levels of serum NGAL and Kim-1 were also dramatically increased. The concentrations of Scr, BUN, NGAL and Kim-1 varied with different interventions.

Changes in the text:(see Page 8, line 247-253).

17. Author has mentioned that protective effect of Lip-1 and Klotho was diminished after Nrf2-IN-1 treatment throughout manuscript. This statement cannot be confirmed because there was no group with combinational treatment of Lip-1 and Nrf2-IN-1 reported. Please edit this statement to only include confirmation about Klotho and not Lip-1.

Reply 17: We have re-refined the language and changed inaccurate narratives in the article.

18. For figure 1-4, the fluorescent images presented are really small and not visible enough to verify the result statements. Please split figures so that each figure is visible to readers.

Reply 18: We've re-edited the image.

19. Please include enlarged images of the electron microscopy.

Reply 19: We've re-edited the image.

20. Please describe in more detail Figure 2E,2G.

Reply 20: This study further clarified the temporal expression of GPX4 and Nrf2 in renal tissue 24 hours after CLP model preparation using immunofluorescence method, which is similar to the results of WB; And clarified the localization of GPX4 and Nrf2 in renal tissue: mainly expressed in renal tubules.

Changes in the text:(see Page 9, line 289-292)

21. Line 283-285 the statement regarding the Nrf2 inhibiting ferroptosis cannot be made as per the results reported.

Reply 21:We modify the relevant narrative to confirm our claim as a whole according to Figure 2 (see Page 9, line 297-300)。

Changes in the text: The above findings suggest that the mechanism of action of Klotho is to nephroprotective by inhibiting the iron apoptosis signaling pathway through the activation of Nrf2 (Figure 2I-J), which effectively reduced the iron content in the tissues (Figure 2K).

22. Line 285 please complete the sentence.

Reply 22: We have made some editing in the text (see Page 9, line 297-300).

Changes in the text: The above findings suggest that the mechanism of action of Klotho may be nephroprotective by inhibiting the iron apoptosis signaling pathway through the activation of Nrf2 (Figure 2I-J), which effectively reduced the iron content in the tissues (Figure 2K).

23. Line 290 please define WB. It is hard to make the statement reported between line 292-293 from the western blot reported. Please edit. Also please split this long sentence.
Reply 23:We have added a definition of wb and a more detailed description of the conclusions obtained (see Page 10 line 304,Page 7, line 223)。
Changes in the text: Western blot analysis (WB) is an antibody-based protein analysis technique; Klotho requires a special secondary antibody, which is incubated with 5ug/ml of recombinant Klotho and then conjugated to a chemiluminescent triple antibody for visualization.

24. Line 337 LPS does not induce actual sepsis, it is a model of sepsis
Reply 24: We have made changes (see Page 11, line 351-353)
Changes in the text: Recently, researchers have started exploring new diagnostic and therapeutic approaches for SA-AKI based on relevant genetic biological characteristics.

25. Page 11 Line 358 “bloodo knoc barrier” -?
Reply 25: We have made changes (see Page 12, line 375)
Changes in the text: Klotho knockout mice are prone to blood-brain barrier damage and central nervous system disorders.

26. The discussion is lacking information about Lip1.
Reply 26: We’ve made additions to the article (see Page 8, line 238-241)
Changes in the text: Lip-1 is a specific inhibitor of iron death, and some studies have confirmed that exogenous supplementation of Lip-1 ameliorates lung tissue damage in acute lung injury induced by CLP and LPS. However, as a chemical compound, there are no studies and evidence for clinical use for the time being.

27. Figure 1 legend, please include details about Fig. 1A-H For example, “Assessment of renal function using (A) Serum Creatinine (Scr) (B) Blood Urea Nitrogen (BUN) analysis. ELISA analysis of renal injury markers (C) NGAL, (D) KIM-1”. What was the source of the NGAL, KIM-1 measurements? Serum, urine, or tissue? Follow the same for all figure legends.
Reply 27: The source of the measurements was serum, which we have modified in the article (see Page 17, line 554-557).

28. Font sizes within figures are too small, especially the x-axis below the graphs.
Reply 28: We have enlarged the image.

29. Figure 2 – “representative bands” – need to describe the assay used i.e., Western blot.
Reply 29: We havedescribe the assay used. (See Page 18, line 565-569).

30. Running title – “ameliorates” not “ameliorate.”
Reply 30: We have made some changes in the text (see Page 1, line 16).

Changes in the text: Klotho ameliorates acute kidney injury.

Reviewer B

The paper titled “Klotho activation of Nrf2 inhibits the ferroptosis signaling pathway to ameliorate sepsis-associated acute kidney injury” is interesting. In the SA-AKI model, Klotho attenuated renal tissue injury, increased HK2 cell viability, decreased inflammatory factor expression and oxidative stress, restored tubular epithelial mitochondrial function, and increased its level in circulating blood, renal tissue and HK2 cells. Klotho probably exerts its protective effects by activating Nrf2 to inhibit the ferroptosis signaling pathway. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) Please analyze the mechanism of ferroptosis regulation in AKI based on the results of this study and in combination with relevant literature.

Reply 1: Klotho reduces the accumulation of lipid peroxides and iron ions in tissue cells by inhibiting the release of inflammatory factors, attenuating the degree of oxidative stress, and improving the homeostasis of the tissue and intracellular environments, thereby inhibiting iron death signaling discussions and ameliorating tissue and cellular damage.

2) The morphological figures in the results of this study are generally small and difficult to see clearly. Please upload clearer figures again.

Reply 2: We’ve made changes.

3) The biological characteristics of ferroptosis-related genes and its research progress in AKI should be added to the discussion.

Reply 3: Recently, some researchers have been exploring new ideas for the diagnosis and treatment of SA-AKI by looking at the biological characteristics of the related genes.

4) The epidemiology and pathophysiology of SA-AKI should be increased, and data related to diagnosis and treatment strategies should be reviewed and analyzed more extensively.

Reply 4: Relevant discussion descriptions have been added.

5) How to provide candidate targets for the treatment of SA-AKI based on the results of this study? It is recommended to include relevant descriptions in the discussion.

Reply 5: Based on the results of the present study, it is suggested that iron death plays a key role in SA-AKI, and the validation of the relevant biological mechanism of action of Klotho may prioritize the use of drugs with iron death inhibition for septic patients in future clinical management to reduce renal injury in patients.

6) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “Transcriptomic analysis and laboratory experiments reveal potential critical genes and regulatory mechanisms in sepsis-associated acute kidney injury, PMID: 35957725”. It is recommended to quote the articles.

Reply 6:Already cited in the discussion section（see Page 11,line 351-353）.

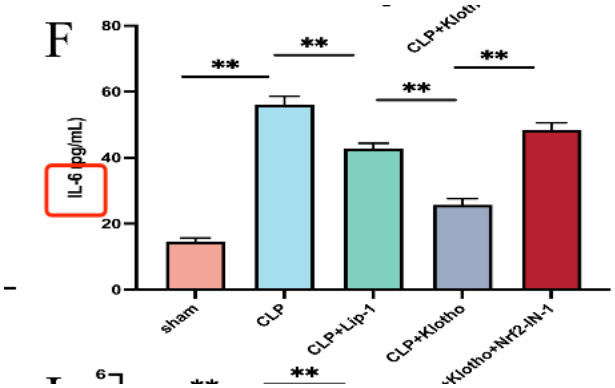
7) It is suggested to increase the possible mechanism of this therapy in the discussion section, so as to enrich the information of this paper.
Reply 7:Relevant narratives have been added to the article（see Page 13,line 431-438）.

Reviewer C

1. **Figure 1**

a) As there are no symbols *** and **** in the figure 1, please delete the explanation in the legend.
b) Please check if the figure matches the legend.

(C,D) Levels of NGAL (C) and Kim-1 (D) in serum. n=6. Levels of TNF-α (E) and IL-6β (F) in serum. n=6. Levels of GSH-Px (G), T-SOD (H) and MDA (I) in serum. n=6.



Reply: We have revised the article.

2. **Figure 2**

a) As there is no symbol ** in the figure 1, please delete the explanation in the legend.
b) Please explain what is the L mean.

under an electron microscope (scale bar =1 μm, 5 μm) (C). (n=3). (L) Morphological

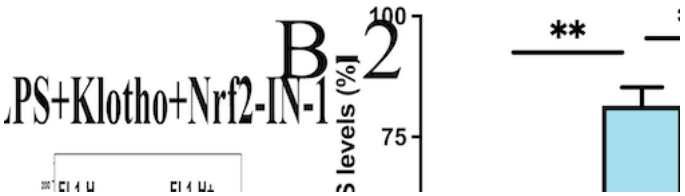
Reply: We have revised the article.

3. **Figure 3**

a) Please also provide the description of 3C in the legend.
c) Please define WB in the legend.
Reply: We have revised the article.

4. **Figure 6**

a) The words are too close, please revise.



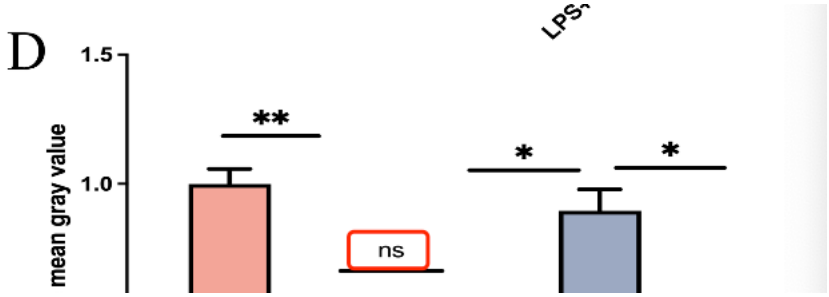
b) There are no J, K, L, and M in the figure, please check.

611 control. (J) Representative IF images and (K) quantification of GPX4 (red) in HK2 cells
612 in each group (scale bar =50 μm). n=3. (L) Representative IF images and (M)
613 quantification of Nrf2 (red) in HK2 cells in each group (scale bar =50 μm). n=3. The
614 results are expressed as the means ± SDs. *, P<0.05; **, P<0.01. LPS,

Reply: We have revised the article.

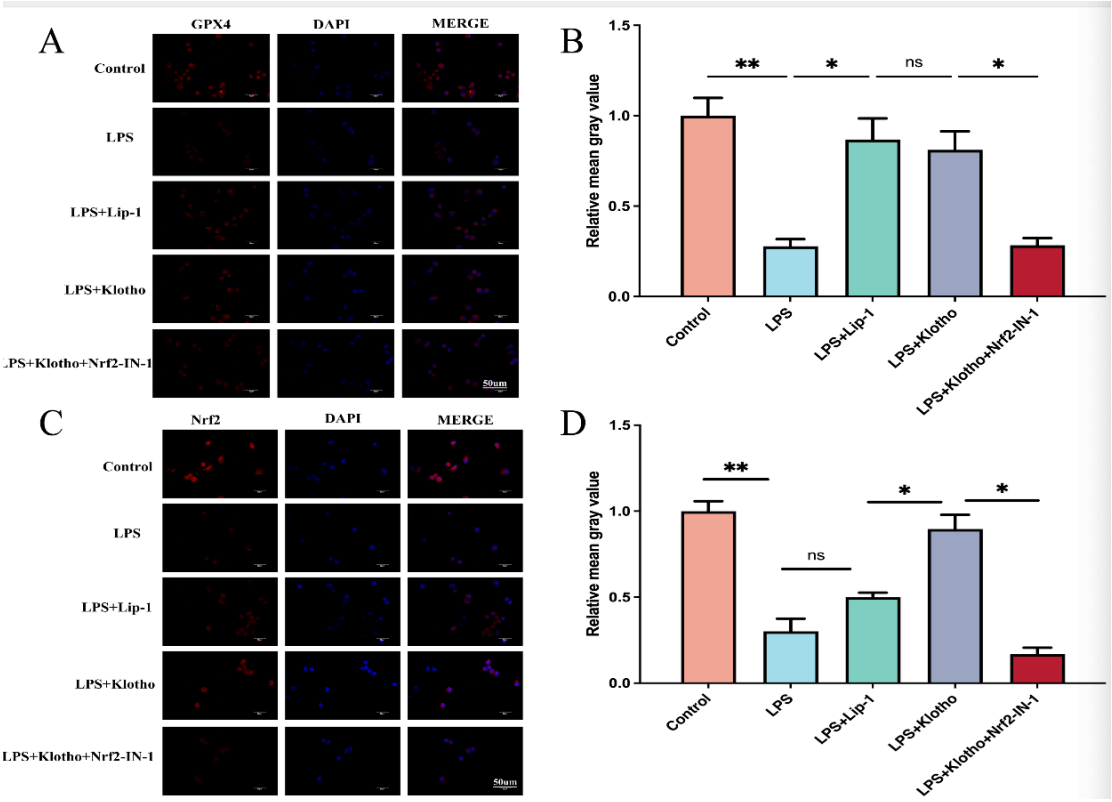
5. Figure 7

a) Please explain ns in the legend.



b) In figure, only A-D were included, no E-H, please check.

342 that in the Klotho group (Figure 7E-F). The immunohistochemical assay results were
343 consistent with the WB results(Figure 7G-H).



Reply: We have revised the article.

6. Figure 8

a) Figure 8 was not cited in the main text, please revise.

b) Please explain ns in the legend.

Reply: We have revised the article.

7. References/Citations

a) Please double-check if more studies should be cited as you mentioned “studies”. OR use “study” rather than “studies”.

98 biochemistry, histomorphology, and genetics (7). Previous studies have shown that
99 ferroptosis is a key mechanism for sequential tubular cell death after ischemic kidney
100 injury and that inhibition of the ferroptosis signaling pathway can promote kidney
101 recovery and reduce the severity of kidney injury (8). There are no relevant studies on

b) Please double-check if citations should be added as you mentioned “studies”.

238 mice. Lip-1 is a specific ferroptosis inhibitor, and studies have shown that exogenous
239 supplementation of Lip-1 improves lung tissue damage induced by CLP and LPS in

364 functional damage and tissue cell death (27). An increasing number of studies suggest
365 that modulation of the ferroptosis signaling pathway may be a new and effective
366 therapeutic target (21). In this study, the presence and important role of ferroptosis in

Reply: We have revised the article.