Peer Review File

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Review Comments

The paper titled "Involvement of UPRmt and ATF-4 in the mitochondrial damage pathway of BEAS-2B cells induced by cigarette smoke extracts" is interesting. Cigarette smoke extract caused mitochondrial dysfunction and morphological changes, and CSE altered the expression of Tim23 and ATF-4, suggesting that CSE may cause mitochondrial damage through the UPRmt and ATF-4 pathways. However, there are several minor issues that if addressed would significantly improve the manuscript.

Tim23 is a core component of the mitochondrial endomembrane transposition enzyme complex, and its upregulation may be a downstream response to UPRmt activation, but it is not a direct or specific marker of UPRmt activation. The conclusion of UPRmt activation based solely on upregulation of Tim23 protein expression is insufficient and not specific enough. At least 1-2 recognized and more direct UPRmt markers need to be detected to more reliably support the conclusion of UPRmt activation.

It is suggested to supplement the current research status on the induction of lung cell damage by CSE and the core role of mitochondrial damage in CSE mediated bronchial epithelial cell damage, in order to highlight the necessity of research.

The current methods alone are not sufficient to fully prove that CSE causes mitochondrial damage. It is necessary to comprehensively assess the decrease in ATP production, inhibition of respiratory chain complex activity, and mitochondrial morphological abnormalities. Suggest adding relevant content.

The full name of the term should be given when it first appears. Please review and revise the entire text.

The current conclusion that suggests CSE may cause mitochondrial damage through the UPRmt and ATF-4 pathways lacks key evidence to support it. The conclusion should be changed to observed phenomena or supplemented with key mechanism validation experiments to establish causal relationships.

The conclusion mentions mitochondrial dysfunction and morphological changes, but there is no mention of any morphological testing in the methods and results section. It is recommended to supplement the methods and results of observing mitochondrial cristae structure and swelling under electron microscopy.

What theoretical basis can this study provide for exploring mitochondrial protectants? It is suggested to explain the potential value of this study for cigarette smoke-related lung diseases and briefly supplement the translational significance of the research.

Comment 1: The conclusion of UPR^{mt} activation based solely on upregulation of Tim23 protein expression is insufficient and not specific enough.

Reply 1: We appreciate the reviewer raising this critical point, which is of great importance to us. We reviewed relevant literature indicating that Tim23 knockdown

disrupts the protein import pathway, stimulates ROS release, and selectively activates the CHOP branch of the UPR. However, the altered Tim23 expression observed in our study does not directly prove activation of the UPR^{mt} pathway, as it is not the sole specific marker for UPR^{mt} activation. We regret to inform you that due to current experimental constraints and funding limitations, we are unable to supplement our study with additional assays for UPR^{mt} markers at this time.

In the revised manuscript, we have adjusted the text and conclusions to describe the observed phenomena rather than asserting definitive activation of UPR^{mt} （ see Page 1, line 20-23） We now indicate that alterations in Tim23 and ATF-4 suggest potential involvement of the UPR^{mt} pathway, which requires confirmation through measurement of more specific markers (e.g., HSP60, CHOP, or ATF5) in future studies. We have also incorporated relevant limitations into the discussion section. We sincerely appreciate the reviewers' valuable feedback and will initiate the suggested research immediately after optimizing our experimental conditions. This will become a key focus of our future work.

Comment 2: It is suggested to supplement the current research status on the induction of lung cell damage by CSE and the core role of mitochondrial damage in CSE mediated bronchial epithelial cell damage, in order to highlight the necessity of research.
Reply 2: In response to this suggestion, we have added information in the introduction regarding the current state of research on CSE-induced lung cell damage and the central role of mitochondrial injury in CSE-mediated bronchial epithelial cell damage to justify the necessity of this study (see page 2, lines 75-86).

Comment 3: The current methods alone are not sufficient to fully prove that CSE causes mitochondrial damage.
Reply 3: We sincerely appreciate the reviewer's critical suggestion, which we fully agree with. A comprehensive assessment of mitochondrial function including ATP production, respiratory chain complex activity, and ultrastructural morphology would provide a more complete understanding of the CSE-induced mitochondrial damage mechanism. However, due to current experimental constraints and time limitations for this revision, we were unable to perform these additional analyses. Nevertheless, our existing data demonstrate reduced mitochondrial membrane potential and increased mitochondrial reactive oxygen species (ROS), which are indicators of mitochondrial oxidative stress and dysfunction—typically early events preceding damage. Therefore, in the revised manuscript, we have corrected statements that “suggest” or “indicate” mitochondrial dysfunction and stress, removed corresponding annotations, and explicitly identified ATP level measurement, respiratory chain complex activity testing, and morphological analysis as key directions for future research in the Discussion section. We sincerely appreciate your guidance.
Changes in the text: In the Discussion section, we highlighted the limitations of the study and explicitly identified ATP level measurement, respiratory chain complex

activity assessment, and morphological analysis as key directions for future research (see page 14, lines 345-355).

Comment 4: The full name of the term should be given.
Reply 4: We thank the reviewer for this important reminder. We have now thoroughly reviewed the entire manuscript and have ensured that all abbreviations are now properly defined upon their first appearance in both the main text and the figure legends.

Changes in the text： We have revised the text throughout the manuscript to define all abbreviations at their first use. A new "List of Abbreviations" has also been added (see Page 19).

Comment 5: The current conclusion that suggests CSE may cause mitochondrial damage through the UPR^{mt} and ATF-4 pathways lacks key evidence to support it. The conclusion should be changed to observed phenomena or supplemented with key mechanism validation experiments to establish causal relationships.
Reply 5: We are extremely grateful to the reviewers for their profound insights into our conclusion. Our current data cannot provide clear mechanism evidence to establish a direct causal relationship. We strictly revised our manuscript in accordance with this suggestion, reconstructing our conclusions around the observed phenomena rather than asserting unproven causal relationships. In the discussion section, we add the discussion of limitations. Further gene-related experiments, such as knockdown or overexpression of ATF-4 and Tim23 to observe their effects on UPRmt and mitochondrial function, are necessary conditions for determining the causal relationship.

Comment 6: The conclusion mentions mitochondrial dysfunction and morphological changes, but there is no mention of any morphological testing in the methods and results section.
Reply 6: We sincerely thank the reviewer for this highly insightful and critical comment. We fully agree that the method of mitochondrial crest structure and swelling through electron microscopy (EM) will provide direct and valuable evidence for CSE-induced mitochondrial morphological damage. However, due to the limitations of experimental techniques and EM equipment during the revision period, we regret that we are unable to conduct these experiments at this time. Therefore, in the revised draft, we changed the expression. We only observed mitochondrial damage. Moreover, in the discussion section, we explicitly acknowledged this limitation and emphasized the key demands for future electron microscopy research(see page 14,lines 345-355). Thank you to the reviewers for their suggestions, which have greatly improved the accuracy of our work.

Comment 7: What theoretical basis can this study provide for exploring mitochondrial protectants? It is suggested to explain the potential value of this study for cigarette smoke-related lung diseases and briefly supplement the translational significance of the research.
Reply 7: Thank you very much for your suggestion. It is crucial to the completeness of

the article. We have added a discussion on the potential value of cigarette smoke-related lung diseases in the discussion section(see page 15,lines 391-398）.

Changes in the text:

Results: The study showed decreased membrane potential and mitochondrial reactive oxygen species （ROS） accumulation in BEAS-2B cells after CSE treatment，this indicated that CSE caused mitochondrial dysfunction and oxidative stress. The expression of Tim23 was up-regulated after CSE exposure, this alteration hints at a potential activation of mitochondrial stress pathways; the expression of ATF-4 showed a positive correlation with the time of CSE treatment, and the expression in the nucleus was increased, which indicated that CSE altered the expression and localisation of Tim23 and ATF-4.

Conclusions: Cigarette smoke extract induces features of mitochondrial dysfunction, and triggers alterations in the expression of Tim23 and ATF-4. These findings are consistent with the model that CSE may impair mitochondrial homeostasis, potentially through mechanisms involving mitochondrial stress responses (such as UPR^{mt}) and ATF-4 signaling.

Discussion: It should be emphasized that our study has limitations. The assessment of mitochondrial damage is multidimensional. To fully elucidate the comprehensive picture of cigarette smoke extract (CSE)-induced mitochondrial damage, more comprehensive assessments are required. These should include measuring cellular ATP levels, evaluating the activity of electron transport chain complexes, and examining mitochondrial cristae architecture and swelling via electron microscopy. These critical studies represent core directions for our future work, aimed at further elucidating the mechanisms by which CSE impairs mitochondrial health.

These findings are consistent with the model that CSE may impair mitochondrial homeostasis, potentially through mechanisms involving We acknowledge the limitations of this study, which failed to further test classical markers of the UPR^{mt} pathway to demonstrate its activation. This will be a key focus of our subsequent work. mitochondrial stress responses and ATF-4 signaling, suggesting that the UPR^{mt} pathway may be involved. Although our research has certain limitations, it provides a potential mechanism for mitochondrial damage in CSE-mediated bronchial epithelial cell damage. Our research also offers a new theoretical framework for the development of mitochondrial-targeted therapies for lung injury caused by CS. Our data suggest that protecting mitochondrial integrity by targeting these specific nodes may be a feasible strategy to alleviate bronchial epithelial damage induced by cs. Our future research will focus on verifying these targets in in vitro and in vivo CS-induced lung injury models using gene (knockout/overexpression) and pharmacological methods to assess their true therapeutic potential.