Peer Review File

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Reviewer A

Comment 1: I think that this manuscript should be submitted to journals for basic experimental studies. It may be difficult for clinicians to understand this study. How will authors use this model?

Reply 1: The reviewer points out that the current study is of preclinical nature. Nevertheless, we consider the findings relevant to readership of Journal of Thoracic Disease as supported by the other reviews. The results of our study are not only of relevance to researchers developing new studies with lung sealants, but will also help the clinical readership to interpret results of current literature of experiments and intervention studies relating to prolonged pulmonary air leakage and lung sealants. We have made a few changes to the manuscript to highlight the importance of our findings for the clinical readership.

Illustrating that the topic of lung sealants are of interest to the clinical public are the results of a recent survey amongst 258 thoracic surgeons of the European Society of Thoracic Surgeons, in which more effective lung sealants were identified as an important unmet need. In addition , a recent systematic review by Aprile et al. showed that the current evidence for lung sealants is inconclusive.

To research and develop these sealants, valid preclinical models are essential, but a translational gap might exist in the present models. To further illustrate this problem, our research group has recently also published a systematic review on animal studies, illustrating that negative control groups to demonstrate the model validity are only used in 18.7% of studies and disease models are only used in 8% of studies. Reduction of this translational gap might directly improve treatment of pulmonary air leakage in clinical practice.

The present study demonstrates which types of lesions are required for an air leak model for lung sealant testing, allowing for further standardization. These findings have been used by our research group in a follow-up study, testing the in-vivo sealing effectiveness of a novel lung sealant.

Changes in text:

- *Introduction, page 4, line 88-99: "Prolonged pulmonary air leak (pPAL) occurs in up to 30% of lung resections, causing increased morbidity (empyema and post-operative complications), reinterventions (4.8%), readmissions (OR = 2) and mortality (OR = 1.9). Preclinical animal studies with lung sealants have shown promising results, and 74% of surgeons use sealants in their high-risk patients. Results of lung sealants in preventing pPAL in clinical studies, however, are mixed and guidelines do not recommend their routine use. Recently, in a European Society of Thoracic Surgeons (ESTS) survey, the majority of the 258 responding thoracic surgeons affirmed the lack of sufficient evidence for lung sealants, and an unmet clinical need for more effective lung sealants was described. The marked discrepancy between the more positive*

preclinical studies and often unsatisfactory clinical results indicate a potential translational gap."

- *Added reference: Aprile V, Bacchin D, Calabrò F, et al. Intraoperative prevention and conservative management of postoperative prolonged air leak after lung resection: a systematic review. J Thorac Dis 2023;15:878-92.*

- *Introduction, page 4, line 104-108: "Furthermore, in contrast to patients undergoing lung resections, animals used in lung sealing experiments are healthy in 92% of cases and may poses enhanced intrinsic sealing and regenerative capacities, which can possibly invalidate positive study findings if unaccounted for. In the present literature, no standardized animal model exists that guarantees clinically significant pPAL, and negative control groups were only used in 18.7% of preclinical studies."*
  - Added reference: Hermans BP, Poos SEM, van Dort DIM, et al. Evaluating and developing sealants for the prevention of pulmonary air leakage: A systematic review of animal models. Lab Anim 2023:236772231164873.

- *Discussion, page 16, line 418-421: "Clinicians and experimental researchers should be aware of the intrinsic sealing mechanisms of healthy animal lungs. Success of sealants in animal studies should therefore be interpreted cautiously. In new experimental design, negative control groups should always be considered to measure the actual treatment effect."*

Reviewer B

Comment 1: Why authors have not tested any sealant in the few animals that showed prolonged air leak after the parenchymal lesion.

Reply 1: The principal aim of the current study was to develop and validate a model that was capable of air leakage for an extended period. The current manuscript is therefore focused on description of lesions suited for further studies and mechanisms of intrinsic sealing. The testing of sealants on these lesions is part of a follow-up study to the current model validation study, which we have performed using a novel lung sealant.

Changes in text

- *Methods, page 6, line 156-157: "For the present study, we only pooled results for untreated lesions, as the focus of this investigation was to study the mechanisms of intrinsic sealing."*

Comment 2: Author should specify that they were studying prolonged air leak in Alveolar-pleural fistula (APF) and not in bronchopleural fistula (BPF) that represent another kind of pathology affecting major airways.

Reply 2: This is indeed an important distinction to define in the present model. Alveolar-pleural fistula (APF) is defined as air leakage arising distal to the segmental bronchus, which has been the case for both the superficial parenchymal lesions and the deeper lesions involving small bronchioles. When considering a surgical scenario, the superficial parenchymal lesions could be compared to air leaks occurring from parenchymal dissections required during segmentectomy or lobectomy. Conversely, the deeper lesions involving small bronchioles in the lung parenchyma could occur when performing non-anatomical resections (e.g. using staplers). Both types of lesions are distinct from lesions affecting the major airways (main stem, lobar, sublobar bronchus) as in broncho-pleural fistula (BPF). We have added this for further clarification in the text.

Changes in text:

- *Methods, page 6, line 160-161: "Lesions were made to simulate the clinical problem of an alveolar-pleural fistula, defined as PAL arising distal to the segmental bronchus."*

Comment 3: Why authors have not tested potential air leak following surgical resection with staplers that are quite common in the surgical daily practice.

Reply 3: Investigations using staplers could be a relevant subject for future studies in a more clinical setting. However, due to the strong intrinsic healing potential observed of the present lesions, we expect stapled lesions to show no relevant prolonged air leaks in healthy animals, as would be the case in patients undergoing lung surgery in diseased lungs (e.g. emphysema). For a rational study design in this case, validation of an animal model of emphysema might be required prior to investigations of air leaks following lung stapling. We further elaborated this in the discussion.

Changes in text:

- *Discussion, page 16, line 424-426: "Such disease models could also be used to study mechanisms of PAL in clinical scenario's, such as those arising from stapler lines."*

Comment 4: How did authors decide the observation period in a back or abdominal position?

Reply 4: In the *post-mortem* studies (in back position), the observation period was kept shorter (minimum of one hour), since no further intrinsic sealing mechanisms were considered plausible in the *post-mortem* situation. In the *in-vivo* situation in abdominal position, a minimal observation period of three hours was chosen. As can be seen in figure 2B, this time span is relevant to observe the initial intrinsic sealing mechanisms. Some variation can be noted (table 1), which is present for practical reasons. For example, if all operative procedures went by rapidly, the observation time could be extended. Longer post-operative observation periods are required for the creation of prolonged air leaks (e.g. in emphysema models), which can be part of a follow-up study. Such a follow-up study requires the rationale of the present study for ethical reasons.

Changes in text:

- *Methods, page 8, line 208-209: "After an observation period in a back position (post-mortem model, minimum of one hour) or abdominal position (in-vivo model, minimum*

of three hours) under mechanical or spontaneous ventilation, the live animals were euthanized using pentobarbital."

Comment 5: Discussion may be enriched by the lecture of the following manuscript: PMID: 36910073

Reply 5: We have added the suggested up-to-date reference to the introduction and discussion.

Changes in text:

- *Introduction, page 4, line 93-94: "Results of lung sealants in preventing pPAL in clinical studies, however, are mixed and guidelines do not recommend their routine use."*
- *Discussion, page 12, line 313-320: "Presently, many clinical studies have been performed, testing numerous different sealant products. Some sealants were shown to be effective, but there were fluctuating results and no clear evidence based recommendations. As we suggest, disappointing results in clinical studies may be a consequence of a translational gap when negative controls are not used in the preclinical phase to ensure significant PAL. For example, effectiveness of fibrin glue was seen in an animal study that compared bursting pressures of fibrin glue with sutures in a rabbit model, without a negative control group (i.e. lesion with no treatment)."*

Reviewer C

We want to thank the reviewer for the elaborated considerations regarding the importance of understanding the mechanisms of intrinsic sealing for further improving the treatment of patients with pulmonary air leakage.

Comment 1: It seems convincing that animal model should give some information to evaluate intrinsic mechanism, however, these models would not explain physiological characteristics in emphysema. Prolong air leakage after pulmonary resections are more frequent in emphysema patients. Taking this account, animal models, the authors developed, would help us understanding the mechanisms of intrinsic sealing in three patterns of lesions.

Reply 1: We agree with the reviewer that the intrinsic sealing mechanisms in the current healthy animal model exhibit different physiological characteristics as compared to patients with emphysema (e.g. see discussion on line 355-361). Further study of intrinsic sealing mechanisms in emphysematous lungs could help us understand these mechanisms, which might help clinical decision making and development of novel treatments. We have further emphasized this in the text.

Changes in text:

- *Discussion, page 14, line 362-364: "Further study of intrinsic sealing mechanisms in emphysematous lungs might help us understand mechanisms of pPAL, which may improve treatment of these lesions."*

Comment 2: We know by experience that there are some mechanisms in wound healing on lesions. The authors clearly explained this in histopathological images in figure 4. I found this very useful as we can eventually classify types of pulmonary lesions. For example, lesions only required superficial coagulations but no fibrin glue, covering or suturing for wound closure. This helps us to consume appropriate sealing agents avoiding over medications, meaning fibrin glue for superefficient damages.

Reply 2: Indeed, these are very relevant considerations raised by the reviewer, describing how understanding of intrinsic sealing mechanisms might help clinicians predict the risk for prolonged air leakage and guide the decision to apply lung sealants accordingly. We have further emphasized this in the discussion.

Changes in text:

- *Discussion, page 16, line 427-429: "With a thorough understanding hereof, a clinical solution for the problem of pPAL might be discovered, for example by making the surgical treatment better synergize with the underlying mechanisms of the specific lesion type."*

Comment 3: I would like to encourage all the authors to continue this study to give us rationale for use of appropriate sealant in pulmonary resections.

Reply 3: We want to thank the reviewer for this encouragement. We have already performed follow-up studies with the present model (not yet published), investigating the *in-vivo* effectiveness of a novel sealant in-vivo.