Peer Review File

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Reviewer A

In this study, the authors have observed that treatment with Astragalus Radix (AR; Huangqi) and Angelica Sinensis Radix (AS; Danggui) protects against idiopathic pulmonary fibrosis (IPF) by inducing autophagy through the inhibition of the mTOR signaling pathway. Furthermore, they show that the treatment with both plant extracts has a synergistic effect and is superior to that of AR or AS alone.

Major concerns:

I have several concerns about this paper. The main one is the study design; the dose of bleomycin used to induce fibrosis is very high, usually lower doses are used. The duration of the study is also too long; normally fibrosis develops from 15 days after bleomycin administration. Drug treatment begins only 2 days after bleomycin administration; it should be noted that this is a pre-treatment for fibrosis, not a treatment. To conclude that plant extracts are effective as a treatment for IPF, their effect should be studied when fibrosis is already established in the lungs, not just 2 days after bleomycin administration when only the inflammatory phase has been triggered. In addition, a group receiving prednisone is used as a positive control for the drug. I find this group questionable as a positive control as prednisone is not a treatment that inhibits pulmonary fibrosis.

Furthermore, I am most concerned about the histological images. Although there is an increase in septal size and some inflammation in the bleomycin-treated groups, there is no clear fibrosis. The Mason's trichrome stain does not show the characteristic colours of this stain. This is all very worrying as fibrosis may not have developed and the results may be inconclusive.

Reply: Really thanks for your professional review. The dosage use of bleomycin and the duration of the study was performed according to several previously published articles (DOI: 10.33549/physiolres.934892, DOI: 10.1038/s41598-017-11450-3). In addition, prednisone as a positive control was also used according to a previously published article (DOI: 10.1056/NEJMoa1113354).

In addition to the H&E and Masson staining, we also conducted the immumohistochemical staining of fibrosis-related proteins, including α-SMA and Collagen I. As results shown in figure 2A-D, AR-AS herb pair significantly inhibited both α-SMA- and Collagen I-stained cells. Results in figure 2E-F showed that, AR-AS herb pair inhibited the expression of fibrosis-related proteins, including α-SMA, Collagen I, Fibronectin, and Vimentin. Altogether, we conclude that AR-AS herb pair could effectively inhibited lung fibrosis.

Changes in the text: None.

Reviewer B

The paper titled “Improvement of idiopathic pulmonary fibrosis through a combination of Astragalusradix and Angelica sinensisradix via mammalian target of rapamycin signaling pathway-induced autophagy” is interesting. The combination of AR and AS protects against IPF by inducing autophagy via inhibiting the mTOR signaling pathway. The synergistic action of AR and AS is superior to that of either AR or AS alone. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) There have been many studies on idiopathic pulmonary fibrosis. What is the difference between this study and previous studies? What is the innovation? These need to be described in the introduction.

Reply: We have mentioned the innovation in the introduction section. Please see the last paragraph the introduction section.

Changes in the text: However, the mechanism of AR and AS in ameliorating IPF has not been clearly established. In this study, we investigated whether the AR-AS herb pair protects IPF rats from lung inflammation and fibrosis and whether it can regulate intracellular collagen deposition by inducing autophagy. (line 98-103).

2) The description of some methods in this study is too simplistic, please describe in detail.

Reply: We have tried our best to provide more detailed description of the method section. Please check.

3) There are many detection methods for autophagy and inflammation. If multiple methods are used, the results may be more reliable. It is suggested to add test results of other methods.

Reply: Thanks for your professional suggestions. Considering the revision time is limited, we did not test these results using other methods. We will do so in our following studies according to your kindest suggestions.

Changes in the text: None.

4) The abstract is not sufficient and needs further modification. The research background did not indicate the clinical needs of the research focus.

Reply: We have modified the abstract section as you suggested.

Changes in the text: There is a major need for effective, well-tolerated treatments for idiopathic pulmonary fibrosis (IPF) in clinic. (line 35-36)

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

Reply: Autophagy and inflammation related genes might be potential targets for the treatment of idiopathic pulmonary fibrosis, as AR-AS herb pair could significantly regulate autophagy and inflammation. We have discussed the relationship between AR-AS herb pair and autophagy/inflammation in the discussion section.

Changes in the text: None.

6) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "The oncogenic landscape of the idiopathic pulmonary fibrosis: a narrative review, Transl Lung Cancer Res, PMID: 35399571". It is recommended to quote this article.

Reply: Thanks for your kindest suggestion. The article you mentioned above has been cited in the main text as ref [5].

Changes in the text: Unfortunately even those who do receive a lung transplantation, have a lower survival time compared to those who receive lung transplantation for other types of underlying lung diseases (4, 5). (line 75)

7) What is the potential application value of AR and AS in clinical practice? What is the basis for selecting the concentration of AR and AS in this study? Is the dosage safe in clinical practice? Please provide literature support.

Reply: The concentration of AR and AS used in animal studies was selected according to the following articles: DOI: 10.1371/journal.pone.0180417, DOI: 10.1155/2017/9786972. The concentration of AR and AS used in cell studies was selected according to an article: DOI: 10.1186/s12906-019-2781-4. In this present paper, we preliminary demonstrated the therapeutic effect of AR-AS herb pair on treating idiopathic pulmonary fibrosis. The potential application of AR-AS herb pair in clinic should be confirmed based on much more experiments. We will continue study the therapeutic effect of AR-AS herb pair in the following to address your concern. Thanks again for your technique review.

Changes in the text: None.

Reviewer C

1) First, the title needs to indicate that this is an animal study.

Reply: Thanks for your kindest suggestions. As you suggested, the article title has been revised.

Changes in the text: Improvement of idiopathic pulmonary fibrosis through a combination of Astragalus radix and Angelica sinensis radix via mammalian target of rapamycin signaling pathway-induced autophagy in rat. (line 2-4)

2) Second, the abstract needs some revisions. The background need to describe the potential clinical significance of this research focus and what the current knowledge gap is. The methods need to describe the number of rats and how they were grouped. The results need to quantify the findings by reporting the outcome values and accurate P values to support these findings. The conclusion needs comments for the clinical implications and the limitations of this study.

Reply: We have added the the potential clinical significance as you suggested in the abstract section. We have described the number of rats and how they were grouped in the abstract section. However, considering the abstract limited 250 words, we did not include outcome values and accurate P values in the abstract section.

Changes in the text: Background: There is a major need for effective, well-tolerated treatments for idiopathic pulmonary fibrosis (IPF) in clinic. Astragalus radix (AR; Huangqi) and Angelica sinensis radix (AS; Danggui) have been frequently used in the treatment of IPF. This study aimed to reveal the pharmacological effects and the mechanisms of the action of an AR-AS combination in treating IPF.

Methods: Sprague Dawley rats were randomly divided into six groups (n=5): sham, bleomycin (BLM) model, AR, AS, AR + AS, and prednisone (PDN) groups. A transforming growth factor-β1 (TGF-β1)-induced MRC-5 cell model were also used. Pulmonary fibrosis, inflammation, oxidative stress, and autophagy were evaluated by performing hematoxylin and eosin staining, Masson staining, immunohistochemical staining, quantitative real-time polymerase chain reaction, Western blotting, enzyme-linked immunosorbent assay, immunofluorescence, and hydroxyproline assay following the treatment of AR, AS, and the AR-AS herb pair. (line 35-47)

3) Third, in the introduction of the main text, it is important to provide the clinical evidence that supports the efficacy of AR-AS for IPF. Without this prerequisite, it is questionable whether this experimental study deserved to be done. The authors need to describe the potential clinical significance of this research focus.

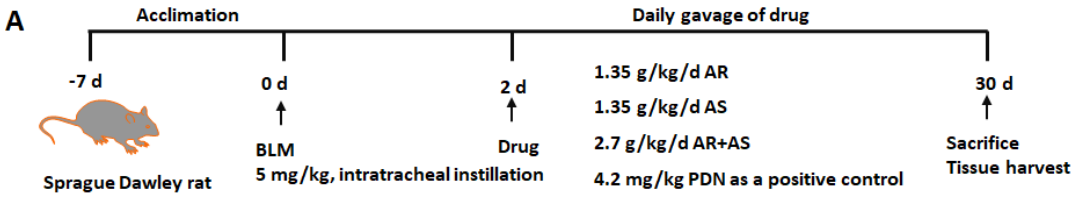
Reply: Thanks for your professional suggestions. We have introduced the clinical applications of AR-AS for IPF in the introduction section.

Changes in the text: Clinical studies on the treatment of IPF used herbal medicine included RA and RAS as the main components and have obtained good curative effect (6). (line 93-95)

4) Fourth, in the methodology of the main text, please use a flowchart to briefly describe the research procedures of this study. In statistics, please describe why Tukey test was used for post-hoc comparisons and ensure P<0.05 is two-sided.

Reply: As you suggested, we have provided a flowchart to briefly describe the research procedures of this study.

We also provided here; so, you can easily track the change.



Two of the most common methods of pairwise comparisons are the Tukey test and the Newman-Keuls test. Both tests are based on the "Studentized range" or "Student's q". The Newman-Keuls test is most frequently used in psychology, while the Tukey test is most commonly used in other disciplines. An advantage of the Tukey test is to keep the level of the Type I error (i.e., finding a difference when none exists) equal to the chosen alpha level (e.g., $\alpha = .05$ or $\alpha = .01$). An additional advantage of the Tukey test is to allow the computation of confidence intervals for the differences between the means. So, here we used Tukey test for the post hoc comparison. In addition, we ensure P<0.05 is two-sided.

Changes in the text: None.

5) Finally, please cite several related papers: 1. Bloem AEM, Mostard RLM, Stoot N, Vercoulen JH, Peters JB, Spruit MA. Perceptions of fatigue in patients with idiopathic pulmonary fibrosis or sarcoidosis. J Thorac Dis 2021;13(8):4872-4884. doi: 10.21037/jtd-21-462. 2. Stella GM, D'Agnano V, Piloni D, Saracino L, Lettieri S, Mariani F, Lancia A, Bortolotto C, Rinaldi P, Falanga F, Primiceri C, Corsico AG, Bianco A. The oncogenic landscape of the idiopathic pulmonary fibrosis: a narrative review. Transl Lung Cancer Res 2022;11(3):472-496. doi: 10.21037/tlcr-21-880. 3. Nogawa H, Matsumoto Y, Tanaka M, Tsuchida T. Diagnostic usefulness of bronchoscopy for peripheral pulmonary lesions in patients with idiopathic pulmonary fibrosis. J Thorac Dis 2021;13(11):6304-6313. doi: 10.21037/jtd-21-1067.

Reply: Thanks for your kindest suggestions. We have carefully studied the articles you mentioned above, and two of them have been cited in the main text.

Changes in the text: IPF is a progressive and lethal interstitial lung disease characterized by a worsening condition of the fibrotic lung changes on chest images,

restrictive spirometric changes, increased dyspnea, and worsened functional status leading to respiratory failure and death (21, 22).

Reviewer D

The manuscript "Improvement of idiopathic pulmonary fibrosis through a combination of Astragalus radix and Angelica sinensisradix via mammalian target of rapamycin" presents an interesting argument for the use of traditional Chinese herbal medicines in the treatment of IPF. The authors speculate on the combination of AR and AS has the ability to reduce extracellular matrix deposition by restoring mTOR-mediated autophagy.

This paper is in general well written and shows some interesting data, mostly observational. There are major limitations however.

Major Issues

1. The major issue here is that the authors did administer the compounds day 2 after bleomycin, but not later. While the effects are respectable, the data only shows that bleo induced fibrosis is prevented, but not treated / slowed or reversed. They should perform experiments in which they administer the compounds not earlier than 7 days after bleo. If they already did these experiments and they turned out negative, they need to downtone the message throughout the paper.

Reply: Really thanks for your professional review. The duration of the study was performed according to several previously published articles (DOI: 10.33549/physiolres.934892, DOI: 10.1038/s41598-017-11450-3). We understand that, it will be better to perform the experiments not earlier than 7 days after bleomycin treatment. However, considering the revision time is limited, we did not do so. We will perform this experiments in our following experiments. Thanks for your kindest suggestion.

Changes in the text: None.

2. It has been shown that bleomycin begins to resolve after approximately 28 days with the peak fibrotic period typically occurring around 21 days. The authors should provide results from other time points (e.g. day 14 and day 21 post bleomycin administration) to ensure that the anti-fibrotic effects seen by AR and AS are not due to the eventual resolution seen in the bleomycin model.

Reply: Thanks for your professional review. As you mentioned, multiple detection times points will further confirm the results of present paper. We will do so in our following studies.

Changes in the text: None.

3. Please explain why statistical comparisons between the AR, AS, and AS+AR groups were only made for the experiments conducted in figure 6. The authors claim that there is a synergistic effect when AR and AS are used together, however, there is no statistical evidence of this in the in vivo and some of the in vitro data.

Reply: As you seen, the effects of a combination of AR and AS is better than AR or AS alone. But, considering we did not compared the effects of AR+AS with AR or AS using statistical analysis, we did not describe the effects of AR+AS using 'significant' in the result section.

Changes in the text: None.

Minor issues

1. The group labelled "sham" in the animal experiments should be changed to "control" to keep consistent with the labels used in figures 1-3. Also, sham is a controversial label typically used in clinical trials.

Reply: We have revised 'sham' by 'control' as you suggested.

Changes in the text: In the control group…. (line 140); control group (line 144-148).

2. Please explain the use of MRC-5 cells in place of primary human lung fibroblasts as they would have been a better example of the native biology.

Reply: MRC-5 is made up of fibroblasts isolated from the lung tissue derived from a White, male, 14-week-old embryo by J.P. Jacobs in 1966. They are also limited passage cells as the same with primary human lung fibroblasts. Many previously published articles have used MRC-5 cells for the research of pulmonary fibrosis. For example: DOI: 10.1016/j.redox.2022.102509, DOI: 10.1186/s40360-018-0204-7, DOI: 10.1016/j.phymed.2023.154680. In addition, compared with primary fibroblasts, commercially purchased MRC-5 cells have several advantages, including high purity and its quality is easy to control. Based on these reason, we used MRC-5 cells.

Changes in the text: None.

3. It would be helpful to include information on how AR and AS are currently being used in the treatment of IPF and other diseases and if the dosage used the experiments detailed here reflect that.

Reply: The clinical use of AR and AS in the treatment of IPF has been introduced as you suggested.

Changes in the text: The AR-AS herb pair has been widely used in clinical practice for patients with IPF (6). Clinical studies on the treatment of IPF used herbal medicine included RA and AS as the main components and have obtained good curative effect (6, 17).

4. Please explain why the specific method of preparation of the AR and AS extracts was chosen.

Reply: In the long history of evolution of traditional Chinese medicine, various forms of herbal preparation had been developed. Among them, decoction in water and ethanol are the two dominant ways to administrate herbal medicine due to their simple application, easy preparation, low cost and proven medicinal efficacy. In this study, AR and AS extracts were prepared by ethanol extraction method.

Changes in the text: None.

5. On line 187 change “Proteins were separated on” to “Proteins were separated using”.

Reply: We have modified this sentence as you suggested.

Changes in the text: Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and…