Author's response to reviews

Title: Dissection of the mechanism of traditional Chinese medical prescription-Yiqihuoxue formula as an effective anti-fibrotic treatment for systemic sclerosis

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Author's response to reviews: see over
Dear Dr. Swaran Flora,

We are so appreciated for your letter about the revision of our manuscript entitled “Dissection of the mechanism of traditional Chinese medical prescription-Yiqihuoxue formula as an effective anti-fibrotic treatment for systemic sclerosis” (MS: 8778042761104917). According to the journal style and formatting guidelines, we have made some revisions in our title page, Abstract section, Authors’ contributions section and References section. As for the reviewers’ comments, a point-by-point description of our responses is as follows, where the reviewers’ comments are in italics and our responses in Times New Roman type. In addition, we have addressed the comments in our revised manuscript.

Thank you so much for handling with our manuscript for us!

**Part 1:**  
**Reviewer #1**

**Question 1:** Introduction section is too long compared to the total length of the paper. Therefore, a shorter introduction is needed. Moreover, a more recent and updated bibliography is mandatory to explain the behavior of fibroblast in SSc (Quiescent, activated and myofibroblasts).

**Answer 1:** Thanks for the reviewer’s suggestion. We have revised the Introduction section. As for the behavior of fibroblast in SSc, our revision is as follows: The “activated” fibroblast and its contractile and secretory counterpart, myofibroblast, are the primary cell types responsible for the persistent production and deposition of ECM. Tissue injury initiates the chronic inflammation generally involving the activation of inflammatory and immune cells which secrete cytokines, chemokine and growth factors. Then resident fibroblasts (quiescent fibroblasts), pericytes, fibrocytes, epithelial and endothelial cells are recruited, activated and finally differentiated into myofibroblasts. The “activated” fibroblast is an intermediate stage between resident fibroblast and myofibroblast. In addition, the “activated” fibroblast and myofibroblast also produce
growth factors and cytokines such as TGF-β and CTGF to support further fibrogenesis\(^{(1,2,3)}\). (Paragraph 2, Introduction section)

*References:

**Question 2:** Cell culture section: No mention about the SSc patients, if skin samples derived from diffuse or limited SSc patients, and also the number of patients and healthy controls included in the study.

**Answer 2:** Thanks the reviewer for pointing out our negligence. In our study, SSc skin tissues were obtained from 3 diffuse SSc patients, and normal tissues were obtained from 3 healthy volunteers. (Cell culture, Material and methods section)

**Question 3:** In the bleomycin-induced dermal fibrosis section: No mention about how the mice were killed (chemically, physically etc).

**Answer 3:** Thanks the reviewer for pointing out our negligence. In our study, the mice were killed by chloral hydrate anesthesia. (Bleomycin-induced dermal fibrosis mouse model establishment, Material and methods section)

**Question 4:** In the preparation and treatment of recombinant TGF-β1 and TCM: The authors use NIH-3T3 fibroblasts, that are a mouse-cell line and SSc fibroblasts derived from human skin. Please justify this choice putting the attention on how the results could be comparable using human primary fibroblasts and mouse cell-line fibroblasts.

**Answer 4:** Basically, the Part 4 results of our study were aiming to justify this concern. Our Part 4 results showed that the phenotypes of NIH-3T3 fibroblasts treated by TGF-β1 were similar to those of SSc fibroblasts. In detail, the transcript levels of ECM genes
from NIH-3T3 fibroblasts, including Col1a2, Col3a1, Ctgf, Tgf-β1, were significantly increased after the induction of exogenous TGF-β1, as well as type I collagen production, which displayed an SSc-like phenotype. Yiqihuoxue treatment could recover them almost to the normal level.

**Question 5:** In the discussion section: please justify the sentence “Astragalus membranaceous attenuated fibrosis because of its immunomodulatory and anti-inflammatory properties”.

**Answer 5:** Thanks the reviewer for pointing out our unclear statement. Basically, Li W et al. demonstrated that the related constituents from Astragalus membranaceous exhibited an anti-inflammatory effect by the inhibitory activity of NF-κB, and the study by Bogatkevich GS et al. showed that the inhibition of NF-κB could down-regulate the expression of CTGF in SSc fibroblasts. We have added the above description to make the sentence “Astragalus membranaceous attenuated fibrosis because of its immunomodulatory and anti-inflammatory properties”. (Paragraph 2, Discussion section)

References:

**Part 2:**

**Reviewer #2:** Authors decribed in vitro and in vivo efficacy of traditional Chinese medicine (TCM), Yiqihuoxue, for SSc. Yiqihuoxue reduced bleomycin induced skin thickness and suppressed in vitro collagen production of SSc derived fibroblasts. Furthermore, Yiqihuoxue suppressed TGF-beta dependent Smad activation as well as
collagen production.

Major concerns

**Question 1:** The data was obviously clear. But, to elucidate efficacy of TCM, comparative study between agents for SSc treatment with (TCM) is required.

**Answer 1:** Thanks for the reviewer’s suggestion. Basically, available treatments for SSc currently are limited and their anti-fibrotic efficacy is also restricted\(^{(1)}\). Thus, it is difficult to find a suitable drug to be used as controls for comparative study of SSc treatment with TCM. In addition, the bleomycin-induced mouse model is a well-established SSc model\(^{(2)}\) so that it can be used to evaluate the efficacy of Yiqihuoxue formula treatment. Furthermore, those indicators for drug efficacy evaluation, including histopathology, dermal thickness, ECM gene expressions and collagen contents, are generally used approaches to investigate the skin fibrosis\(^{(2)}\). Combined with these results, the efficacy of Yiqihuoxue treatment can be positively confirmed.

*Reference:

**Question 2:** Unless the chemical compounds of Yiqihuoxue is defined, this kind of study should be published in journals related with traditional Chinese medicine.

**Answer 2:** Thanks for the reviewer’s suggestion. Yiqihuoxue formula is prescribed according to clinical experience and has showed good anti-fibrotic efficacy in clinical applications. *Astragalus membranaceus* and *Salvia miltiorrhiza*, which are two major components of Yiqihuoxue formula, have been proven in treating fibrosis by many studies\(^{(1,2,3)}\). Moreover, one of the main active components from *Salvia miltiorrhiza*, Salvianolic Acid B, could attenuate liver fibrosis\(^{(4)}\); the related constituent from *Astragalus membranaceus* could attenuate fibrosis by the inhibition of NF-κB\(^{(5,6)}\). Based on those results, our study attempted to dissect the anti-fibrotic mechanism of
Yiqihuoxue formula by molecular biomedical methods and present its advantages, aiming to achieve the goal of TCM modernization and generalization, which is in accordance with the tenet of this journal.

*References:
3. Lü XY, Li M, Weng MW: **Inhibition effects of constituents of Radix Salvia Miltiorrizae on proliferation and procollagen transcription of dermal fibroblasts in systemic sclerosis.** Zhonghua Yi Xue Za Zhi 2007, **87**: 2426-2428;

Minor concerns

**Question 1:** Most of TCM should be replaced to Yiqihuoxue.

**Answer 1:** Thanks for the reviewer’s suggestion. We have revised it throughout the manuscript.

**Question 2:** Western blotting is unconvincing Figure 5 because figures are artificially
Answer 2: Thanks for the reviewer’s question. Actually, not only the samples with different treatments have been run in the same gel, but also three assays with the same treatment run in the neighboring wells. In addition, the anti-pSmad3 antibody has a heavy background and some non-specific bands. In the revised version, we have provided original figures without cropping and specific bands have been marked.

Question 3: Why authors did not described smad phosphorylation in Figure 3.
Answer 3: Thanks for the reviewer’s question. Actually, the optimal timing of phosphorylation detection is 30 min to 60 min after treatment. In Figure 3, the protein samples were harvested 48 h after Yiqihuoxue treatment, when the phosphorylation changes were hardly detected. However, 60 min is too short for Yiqihuoxue treatment to work. That is also the reason that NIH-3T3 fibroblasts were pretreated with Yiqihuoxue treatment for 24 h followed by TGF-beta induction for 60 min to detect the phosphorylation changes.

Sincerely,
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