Peer Review File

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Reviewer A

1. Please explain why apoptosis was detected at 48 hours instead of 72 hours?
Reply 1: The results of previous CCK8 studies showed that YYR could inhibit the proliferation activity of A549 cells in a concentration and time dependent manner. Since the 72h IC50 lethality of YYR at 0.5mg/ml and 1mg/ml exceeded 50%, YYR at 0.5mg/ml and 1mg/ml for 48h was selected as the experimental conditions.
Changes in the text: see Page 8, line 13-15.

2. Is it appropriate to use GAPDH as an internal parameter for the detection of metabolism-related proteins by WB?
Reply 2: Western Blot experiment has been re-completed, we have changed the internal parameter to β-actin.
Changes in the text:None.

3. cck8 presentation is best expressed in terms of cell viability or inhibition rate.
Reply 3: This study has completed the CCK8 test and improved the relevant test content.
Changes in the text: We have modified our text as advised (see Page 4, line 5-12).

Reviewer B

This manuscript explored the inhibitory effect of Yiqi Yangjing recipe, a traditional Chinese medicine compound, on A549 cells, and preliminarily explored its mechanism. There are some defects in the manuscript which need to be supplemented or modified by the author.
1. The author's description of the preparation process of YYR is very brief. Here's how the authors describe it: "These herbs were powdered and mixed (thewire 10:3:5: 5:10:10:3) the prior to being decocted 8 times in water for 1 hour." Does it mean that all the Chinese herbs were first made into powder and then boiled in water? And I can't understand what the author said: "being decocted 8 times in water for 1 hour". Does that mean it was boiled in water eight times in an hour? Did the author end up preparing YYR's freeze-dried powder? This section needs to be revised and describe the drug preparation process in detail.
Reply 1:These herbs were powdered and mixed (ratio 10:3:5:5:10:10:3), boiled with water repeatedly for eight times within one hour, filtered and concentrated, centrifuged

and added with the same volume of ethanol to the superserum, frozen and dried, filtered with a filter to remove bacteria to prepare YYR extract, and stored at 4℃. The YYR extracts was mixed with the medium, filtered by filter, and prepared into YYR extracts with concentrations of 1, 0.5, 0.25mg/mL for experiments.
Changes in the text: See Page 4, line 29-32 and Page 5, line 1-2.

2. In the section of "Cell Counting Kit-8 assay", the authors did not use a drug-only control group that contained no cells. The experimental principle of CCK-8 detection is based on the change of mitochondrial respiratory activity, that is, the intracellular reducing agent can reduce the triazole tetrazolium (WST-8) in CCK-8 reagent to colored products. As far as I know, most traditional Chinese medicines have colors, especially those that have been boiled in water. The author needs to set a control group containing only YYR and no cells to exclude the influence of the color inherent in YYR on the absorbance value.
Reply 2: The original experiment set YYR (0-1 mg/mL) group without cell suspension, which was described in detail in the article
Changes in the text: See Page 5, line 11-13,15-16.

3. In experiments with TUNEL staining, authors should describe in detail how positive cells were counted. Was it counted by Image J software? Or did the author count them manually?
Reply 3: TUNEL-positive points are calculated using DAPI and TUNEL positive points through image J software. The positive points of DAPI were calculated as the total number of cells, the positive points of TUNEL were calculated as the tunel points number of cells,the ratio of TUNEL/DAPI×100% as apoptotic cells was calculated.
Changes in the text: See Page 7, line 27-31.

4. All Western-blot images in the manuscript need to be indicated molecular weight on the right.
Reply 4: Relevant content has been added to the original.
Changes in the text: See Figures 2-5.

5. The discussion section is too simple and needs to be revised in detail to increase the academic level.
Reply 5: Relevant content has been added to the original.
Changes in the text: See Page 10, line 20-30 32-33, and Page 11, line 1-7.

Reviewer C

The paper titled “Yiqi Yangjing recipe stimulates apoptosis while suppressing the energy metabolism via under-expression of PFKFB3 in A549 cells” is interesting. This study demonstrated that YYR promoted lung cancer cell apoptosis and inhibited energy metabolism by targeting PFKFB3. Furthermore, we believe that YYR may be a suitable supplement or alternative drug for lung cancer treatment. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) What is the potential application value of this agent in clinical practice? What is the basis for selecting the concentration of YYR in this study? Is the dosage safe in clinical practice? Please provide literature support.

Reply 1: The results of previous CCK8 studies showed that YYR could inhibit the proliferation activity of A549 cells in a concentration and time dependent manner. Since the 72h IC50 lethality of YYR at 0.5mg/ml and 1mg/ml exceeded 50%, YYR at 0.5mg/ml and 1mg/ml for 48h was selected as the experimental conditions.

Changes in the text: See Page 8, line 13-15 and Page 10, line 9-12.

2) How can the accuracy of its efficacy be demonstrated since a positive control was not used in the design of this study? What is more appropriate if a positive control is added? What are the similar effects or mechanisms of YYR compared to positive controls? What are its own characteristics? Suggest adding relevant content.

Reply 2: PFK15 (PFK-015) is a potent selective 6-phosphofructo-2-kinase (PFKFB3) inhibitor. In this study, PFK15 was selected as a positive control. YYR has a similar effect compared to PFK15. It was observed that YYR inhibited the glycolysis of human lung adenocarcinoma cell A549, which may be because the prescription controlled the energy uptake of lung adenocarcinoma cell by inhibiting the expression of HIF-1α and PFKFB3 in the glycolysis pathway of lung adenocarcinoma cell, thus inhibiting its proliferation process.

Changes in the text: See Page 8, line 13-15 and Page 10, line 9-12.

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

Reply 3: Apoptosis test and CCK8 test have been completed in this study, and the apoptotic effect of YYR on lung cancer A549 has been proved.

Changes in the text:None.

4) It is suggested to increase the research on cell migration, which may make the whole research more complete.

Reply 4: Apoptosis test and CCK8 test have been completed in this study, and the apoptotic effect of YYR on lung cancer A549 has been proved.

Changes in the text:None.

5) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “Downregulated RPL6 inhibits lung cancer cell proliferation and migration and promotes cell apoptosis by regulating the AKT signaling pathway, J Thorac Dis, PMID: 35280491”. It is recommended to quote the article.
Reply 5: Relevant references have been cited.
Changes in the text: See Page 11, line 10-12.

6) It is suggested that the addition of bioinformatics and network pharmacology methods to analyze key regulatory genes may make the whole study more complete.
Reply 6: Relevant content has been added to the original.
Changes in the text: See Page 4, line 12-26.

7) What is the prognostic role of glycolysis for cancer outcome? It is recommended to add relevant content.
Reply 7: Relevant content has been added to the original.
Changes in the text: See Page 10, line 32-33, and Page 11, line 1-7.

Reviewer D

1. Drug-containing serum was recommended for experiments in vitro
Reply 1: The drawbacks of TCM serum pharmacology are as follows: the optimal administration regimen cannot be determined; The concentration of drugs in vitro culture system cannot be equal to that in blood without affecting the growth of tissues and cells; The optimal blood collection time is difficult to determine. The composition of serum is complex, there are too many uncontrollable factors, and the repeatability of experimental results is poor. The active ingredients inherent in serum often affect the test results, and there is no ideal measure to remove the influence of serum.
Changes in the text:None.

2. Why do not use A549 overexpressing PFKFB3 cells in vivo?
Reply 2: This experiment proved the initial effect of YYR. Due to the insufficient experimental budget and the problem of stable transfection efficiency of overexpressed cells used in vivo, the subsequent selection of gene knockout mice with sufficient budget would make gene expression more stable and the experimental results more reliable.

Changes in the text:None.

3.The background of the WB strips have been adjusted too clean?

Reply 3: Western Blot experiment has been re-completed.

Changes in the text:None.

4. Ethical numbers shall be provided for animal experiments

Reply 4: The ethical code for animal experiments is KS(Y)1804, relevant attachments have been uploaded.

Changes in the text:None.

5.Fig5: "YYR promoted apoptosis while inhibited PFKFB3 expression", "while" should be considered?

Reply 5: We can speculate that YYR may promote apoptosis by inhibiting the expression of PFKFB3.

Changes in the text:None.

Reviewer E

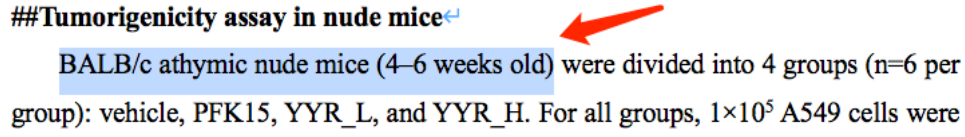
1. Abstract

Please extend the content of the Background. This paragraph should contain 'study background' and 'study objective'.

Reply 13:The relevant content has been modified.

2. Animal's source

Please indicate the source of BALB/c athymic nude mice (bought from where).



Reply 14: Relevant content has been added.

3. Reference/citation

a. There are totally 33 citations in the main text, but there are 34 references in the reference list. Please check and revise.

Noted: *References should be cited consecutively and consistently according to the order in which they first appear in the text.*

Reply 16a:The relevant content has been modified.

b. Please check if more citations should be added in below sentences. Otherwise, 'a study has/a clinical study has' would be more appropriate.

Studies have reported that blocking PFKFB3 significantly promotes the apoptosis of tumor cells (15).

Clinical studies have found that YYR has an inhibitory effect on the proliferation, invasion, and metastasis of lung cancer cells in patients (16).

Reply 16b:Thanks, I already know this. Just say it according to the above statement.

4. Figures 2 and 4

Please indicate the full term of "FCCP" in the figure legend.

Reply 17:The relevant content has been modified.