Peer Review File

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Reviewer Comments

The authors reported the efficacy of Yin Zhi Huang (YZH) on obesity in 2020 (Ann Transl Med. 2020 Mar; 8(5): 231). In the report, they used HFD (Research Diets D12492) in 6 week-old C57BL/6J mice and hypothesized the mechanism of action through decreasing DNL and increasing mitochondrial FAO. In the current manuscript, they used HFD (Research Diets D12492) plus fructose/glucose water in 6 week-old C57BL/6J mice and tried to induce liver fibrosis as differentiation from their previous publication.

I reviewed this manuscript and below is the comment for the author.

Comment 1. The author selected a higher dose of YZH they used in their previous report in 2020. In the paper, a lower dose (10mL/kg decreased SREBP1 and FAS expression in the liver, and data suggested the dose already has an impact on the liver. I want to know why the author selected the dose of 30mL/kg in the current manuscript.

Reply 1: Thank you for your professional advice. Because in our previous study, the 30 ml/kg dose had better phenotype results and more effective in reducing body weight and liver steatosis. At the same time, in our research paper published in 2020, the 10 ml/kg dose could not significantly reduce AST level, while the 30 ml/kg dose protected liver function better. We divided the daily dose of Yinzhihuang into three times, and each gavage dose did not exceed the IACUC regulations. we also did not observe any related adverse events after gavage. And our study was approved by the Zhongshan hospital animal ethics committee.

Comment 2. Related to comment 1, the authors argued that YZH could be a new strategy for preventing NASH development and progression. Comment on the rationale that the dose of 1.35mL/mL YZH is translatable to human patients will be helpful to consider future YZH therapy.

Reply 2: Thank you for your professional advice. Since the human diet is more complex and at the same time there are more factors affecting the human metabolic syndrome, we believe that clinical studies with sufficient sample size are still needed. We are grateful for your constructive comments to us, and we will follow up with a clinical study on the treatment of NASH with Yinzhihuang Oral Liquid. It is noteworthy that the clinical efficacy study of Yinzhihuang oral liquid for the treatment of NASH published in the World Journal of Integrative Medicine, Vol. 10, No. 11, 2015, was conducted at a dose of 20 ml each time, 3 times daily for 12 weeks.

Comment 3. In addition, 30mL/kg of oral dosing is quite high in mouse repeated dosing studies. Based on the body weight data in Figure 1, the initial actual dose volume was 0.75 mL to 1.35 mL (saline or YZH) every day. Normally, the maximum dosing volume is 10mL/kg in mice on an IACUC policy.

Reply 3: Thank you for your professional advice. Please forgive our lack of precision in the description of the method section regarding the dose of gavage in mice; in our study, we divided the daily dose of Yinzhihuang into three equal parts and treated mice three times daily by gavage, and each gavage dose did not exceed the IACUC regulations.

Changes in the text: We have made appropriate changes in the description of the method section (See line 336).

Comment 4. In Figure 1C, the author showed food intake data (g/day). However, I am not sure this is just single-point data or the average. The method mentioned body weight was monitored weekly, but no information of the frequency of food monitoring. Usually, we show all food intake data (or cumulative/total food intake) through the experiment for anti-obesity study because this is very important to address the mechanism of action.

Reply 4: Thank you for your professional advice. We weighed mice food weekly during the study, recorded the weekly intake of mice, and calculated the amount of food taken by each mouse daily. It is an average number, not a single point of data. The details can be seen in table1. Each group contained 6 mice.

Table1. Food intake of mice in each group

Group(g) / Time	ND	HFGD	HFGD+Y
Week1	5.05	5.14	5.69
Week2	4.99	4.29	4.59
Week3	4.93	5.14	4.74
Week4	5.13	5.21	5.37
Week5	5.15	5.29	5.62
Week6	4.67	5.17	4.47
Week7	4.84	4.44	5.55
Week8	5.01	5.08	4.37
Week9	5.15	5.43	5.63
Week10	4.77	4.62	4.98
Week11	4.95	5.11	5.09
Group(g) / Time	ND	HFGD	HFGD+Y
Week12	5.01	5.03	5.45
Week13	4.84	4.99	5.86
Week14	5.04	4.82	4.68
Week15	5.21	5.15	5.64
Week16	5.12	5.09	5.43

Comment 5. It is interesting that 30mL/kg YZH almost completely blocked body weight increase without affecting food intake. As you expected, the total calorie intake between HFGD and HFGD+Y is a huge difference if we calculated the calorie in the diet. It means a huge induction of daily calorie consumption must be happened in the HFGD+Y group compared to HFGD. It is great if the author shares any evidence of increased energy expenditure, body temperature, or activities.

Reply 5: Thank you for your professional advice. We are very sorry that we did not monitor energy expenditure, body temperature, or activities in the study. We will include these aspects in future experiments and thanks a lot once more.

Comment 6. Another possibility of body weight loss by YZH could be the inhibition of dietary energy absorption. Is there any evidence of changes in energy loss in the feces?
Reply 6: Thank you for your constructive comments. We are so sorry that we did not have data on this, and we will include these indicators in the next studies.

Comment 7. In addition, any comment on feces will be helpful (such as the shape of feces and any symptoms of diarrhea).
Reply 7: Thank you for your constructive comments. Mice in HFGD+Y group had no diarrhea. The shape of feces among groups had no differences. We removed 24-hour stool of each mouse and retained in -80°C refrigerator for the analysis of bile acid in feces. In order to show you the shape of feces, we photographed the stool in these frozen tubes and also counted the weight of the stool and the number of stool pellets, and found no significant differences in the 24-hour stool weight and number of pellets between the three groups of mice. The details can be seen in the following figure1 and we added a supplement file for the mamuscript.

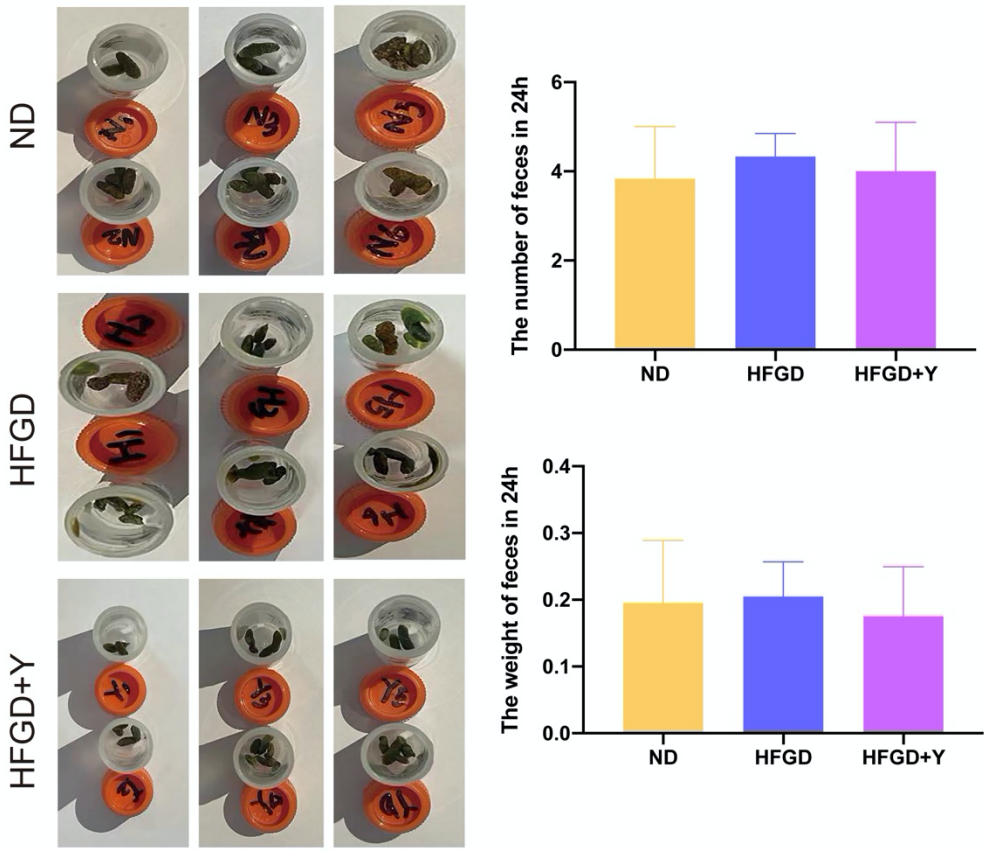

Figure1. Shape and weight of feces, and number of pellets

Comment 8. Just out of curiosity, I also confirm the timing of IPGTT and ITT. If I understand correctly, both experiments were conducted between 16-17 weeks. Could I confirm that these experiments didn't affect the body weight change in Figure 1A? I think these experiments are very stressful in mice because this experiment required fasting, IP injection, frequent tail blood correction.

Reply 8: Thank you for your constructive comments. Please forgive our lack of precision in the description of the method section of our manuscript regarding the timing of IPGTT and ITT. In fact, the last point on the weight curve were collected on Monday of the 17th week, and we began our IPGTT and ITT test on Tuesday and Friday of the 17th week separately. And we sacrificed the mice on Sunday. The body weight of mice collected on Sunday of the 17th week was not used to plot the weight curve.
Changes in the text: We changed "the 16th week of modeling" to "the 17th week of modeling" (See line 390)

Comment 9. In figure 2F, Y-axis uses the SAF score, but the title says NAS. The Y-axis could be NAS?
Reply 9: Thank you for your constructive comments. We are very sorry for the mistake in the text, it is SAF score, not NAS score.

Comment 10. Overall, the authors evaluated multiple outputs using animal samples at the endpoint, but 30mL/kg YZH completely normalized diet-induced obesity in the current study. Thus, all of this evidence might be the result of potent body weight loss.
Reply 10: Thank you for your constructive comments. Certain phenotypic outcomes do exist in animal models induced by high-fat and high-sugar diets that are related with body weight. Blood glucose, ALT/AST, TG/TC and liver pathologies, these OUTPUTS, are positively correlated with body weight.

Comment 11. It is interesting to see that any efficacy induced by YZH could be canceled in germ-free mice (or antibacterial drug co-treatment).
Reply 11: Thank you for your constructive comments. Germ-free mice are the most stringent. In the next experiments, we will design fecal microbiota transplantation experiments with germ-free mice in which feces from mice in the HFGD+Y group will be collected and fed to mice in the HFGD group to study the direct effect of gut microbiota in the treatment of NASH with Yinzhihuang.