Author’s response to reviews

Title: Fructus Ligustri Lucidi Modulates Estrogen Receptor Expression With No Uterotrophic Effect in Ovariectomized Rats

Authors:

Yuqing Tang (tangyuqing@live.com)
Cheng Li (celialee_cheng@163.com)
Xuejiao Sun (1099648128@qq.com)
Yi Liu (18674852163@163.com)
Xiting Wang (wxtlofe@163.com)
Yubo Guo (1264115049@qq.com)
Lili Wang (wangxiaoxing0108@163.com)
Rufeng Ma (marufeng5188@126.com)
Jianzhao Niu (niujianzhao@126.com)
Min Fu (fumin1025@gmail.com)
Dongwei Zhang (zhdw1006@163.com)
Yu Li (liyubeijing1973@163.com)

Version: 2 Date: 16 Sep 2017

Author’s response to reviews:

Response Letter
The Editor,
BMC Complementary and Alternative Medicine

No. BCAM-D-16-01012R1

Dear Editor,

We thank the editor and expert reviewers for considering our submission positively and allowing us to submit the revised manuscript. Now we have finished all the required revisions, and
changes are shown below and also highlighted in green in the revised manuscript. If further concerns are rising, please feel free to contact me.

Regards

Yu Li, MD

Preclinical Medicine School
Beijing University of Chinese Medicine
Beijing 100029, PR China
Phone: (8610) 6428-6374
Fax: (8610) 6428-6929
Email: lliyubeijing1973@163.com

No. BCAM-D-16-01012R1
Fructus Ligustri Lucidi Modulates Estrogen Receptor Expression with No Uterotrophic Effects in Ovariectomized Rats
Yuqing Tang; Cheng Li; Xuejiao Sun; Yi Liu; Xiting Wang; Yubo Guo; Lili Wang; Rufeng Ma; Jianzhao Niu; Min Fu; Dongwei Zhang, Ph.D.; Yu Li, Ph.D.

Response to reviewer 3:

1. Page 5, line 36, what is estrdiol vlerts? Please check the spelling.

Response: Thanks. We are sorry for spelling mistakes. The phrase “estrdiol vlerts” should be replaced by “Estradiol valerate” in the manuscript. We have corrected the spelling errors in the revised version.

Estradiol valerate (EV) is a synthetic steroidal estrogen. It acts as a prodrug of estradiol, and has the same medical uses as those of estradiol and other estrogens.

2. Why did the authors choose to use the dosage (3.5g/kg/day) of FLL in this study? Would it be too high for the rats?

Response: Thank you for the question. The dosage of FLL used in the current study had been demonstrated to be effective in preventing the development of osteoporosis in rats (Chinese Traditional and Herbal Drugs. 2016; 47 (7): 1155-1162). In addition, the dosage of FLL in the current experiment is equivalent to the clinical doses of adults used in the treatment of osteoporosis. Also, the similar dose was demonstrated to be effective by different research groups [Chinese Traditional and Herbal Drugs. 2016; 47 (5): 851-856].
3. The age of the rats used in the study was not specified.

Response: Thanks. The rats used in the study were 12-week-old female Sprague Dawley rats. We have added the age of the rats in the revised version.

Female 12-week-old Sprague Dawley rats (200 ± 20 g) were purchased from Beijing SiBeiFu Animal Technology company (license number: SCXK (Beijing) 2014-0037, Beijing, China). (Materials and methods section, Paragraph 4, page 6, line 50).

4. The brand of antibody used in the immunohistochemical staining was not specified. For the staining of uterus, which cell type in exact was used for quantification? What is the IOD value in figure 1,2,4,5 referring to? The quantification method used for staining was not specified.

Response: Thanks. We have added the information in the revised version.

Rat monoclonal anti-ERα antibody (ab3575) and mouse monoclonal anti-ERβ (ab288) were purchased from Abcam (Cambridge, UK). (Materials and methods section, Paragraph 2, page 5, line 21, and Paragraph 8, page 7, line 16).

Glandular epithelial cells were mainly used for quantification in staining of uterus. The IOD value represents integral optical density, which was used to quantify the expressions of ERα and ERβ in Figures (1, 2, 4, 5).

The results of immunohistochemical staining were quantified by Image Pro-Plus software (version 6, SPSS Inc., Chicago, IL, USA) and the integral optical density (IOD) values were recorded. (Materials and methods section, Paragraph 8, page 7, line 31).

5. What is the method used to determine the E2, LH, and FSH levels? In the abstract the authors wrote radioimmunoassay, but in the method section, the authors wrote ELISA. Please be consistent of the method. If it is ELISA, the full name of it should be shown for the first time it is shown.

Response: Thanks. We are sorry for the mistakes. In the current study, ELISA was used to determine the E2, LH and FSH levels and we have revised it in the revision.

The serum levels of estrogen (E2), luteinizing hormone (LH), follicle-stimulating hormone (FSH) were determined by ELISA. (Abstract section, Paragraph 2, page 3, line 22, and Materials and methods section, Paragraph 7, page 6, line 52).

6. Page 9, line 42, what do you mean by 'contribute to prevent the development of menopausal systems'. The English language in writing should be improved.
Response: Thanks. The phrase 'contribute to prevent the development of menopausal systems' means that FLL treatment may have effect on alleviating menopausal systems. However, the original description did not truly reflect the result. Therefore, we have moved this phrase to the section conclusion.

In addition, our findings also demonstrate that FLL has the ability of coordinating LH and FSH levels in circulation, which may contribute to alleviate postmenopausal vasomotor symptoms. (Conclusion section, Paragraph 1, page 12, line 32).

7. Page 10, line 20, is 'OVX insults' the proper way to use?

Response: Thanks. The phrase ‘OVX insults’ has been replaced by ‘ovariectomy’in the revised version.

8. Page 11, line 49, the regulatory effect of FLL on estrogen receptor does not equal to high affinity to it.

Response: Thanks. We have revised this sentence in the revision.

The results from the current study suggest that FLL may selectively enhance ERβ expression in the uterus of OVX rats. (Discussion section, Paragraph 8, page 10, line 43).

9. Page 11, line 50, 'the application of FLL does not increase the risk of endometrial cancer' is overstated. The result of this manuscript cannot support this conclusion.

Response: Thanks for the critical comments. The word ‘hyperplasia’ has been replaced by ‘cancer’in the revised version.

As compared to other phytoestrogens with high affinities with ERα, the application of FLL does not increase the risk of endometrial hyperplasia. (Discussion section, Paragraph 8, page 10, line 46).

10. In the revised version, the authors add one discussion paragraph of the potential active compounds in FLL, but their effects were on bone turnover instead of estrogenic effect. I think the latter one is the focus of this manuscript. I suggest the authors should make the discussion more focused.

Response: Thanks. We have improved the discussion in the revised version.

In addition, we have demonstrated that FLL water extract mainly includes salidroside, ligistroflavon, acteoside, specnuezhenide, and oleuropein acid [38]. Currently, salidroside was demonstrated to bind to ERα in docking simulation assay [39]. So it is reasonable to deduce that
salidroside may account for the estrogenic like effect of FLL in OVX rats. However, further studies are still needed to identify the contributions of each component in the FLL aqueous extract. (Discussion section, Paragraph 6, page 12, line 8-19).

11. In general, the English language writing in the manuscript needs major revision.

Response: Thanks. We have revised the English language writing in the manuscript. The revised manuscript has also been polished by Hindawi.

Response to reviewer 4:

1. Typo and grammatical errors are noted.

Response: Thanks. We have revised the typo and grammatical errors in the revised version.

2. Sentence phrasing can be further improved so that it is more understandable.

Response: Thanks. We have refined the sentence phrasing in the revised version.

3. Results: when presenting results, it is best to avoid any statement which reflects discussion. It is best to leave the statement in Discussion section.

Response: Thanks. We have reduced the statement in results section according to your suggestion.

4. Results: Need to revise the description in Results. Misleading information regarding Figure 1 and 2. For eg page 10 first paragraph: ER alpha is Figure 1. ER beta is Figure 2. But this sentence mentioned both alpha and beta, and referring to Figure 1. In addition, why did authors say E2 is more significant than FLL? E2 group is not significantly different than FLL. If it is significant, should have put a label to indicate the significant difference.

Response: Thanks for the critical comments. We have revised the description in the section results.

The effect of FLL on ERα and ERβ expressions in the femurs were assessed by immunohistochemical staining. As shown in Figures 1 and 2, ERα and ERβ expressions in the femurs of the OVX control group were significantly decreased (P<0.01), when compared with that of rats in the sham control group. Both FLL and EV treatment significantly increased ERα and ERβ expressions in the femurs of the OVX rats (P<0.05 or 0.01) when compared to that of rats in the OVX control group. (Results section, Paragraph 3, page 9, line 4-15)
5. Some figures only have one significant difference i.e p<0.05 or p<0.01. If only one significant difference exist, then the legend should only mention one. No need to put both significance in the legend.

Response: Thanks for the comments. We have improved the figure legends in the revised version.

6. Figure 6 was not stated anywhere in the text.

Response: Thanks. We apologize for the mistakes. We have improved the description in the revised version.

As shown in Figures 4 and 5, ERα and ERβ were mainly located in the endometrium and glandular epithelia cells, and were highly expressed in the uterus of rats in sham control group and EV treatment group compared to that of rats in the OVX control group as evaluated by immunohistochemical staining (P<0.01). Further, FLL treatment did not increase ERα expression but did obviously increase ERβ expression in the uterus of the OVX rats (P<0.05). These results were also further confirmed by western blotting analysis (Figure 6). (Results section, Paragraph 5, page 9, line 35-48).

7. Discussion page 11: Figure 2 which is the IOD value shows significant difference. But Figure 3, relative ratio, no significant difference for ERbeta. This can be misleading. Thus your sentence 'no obvious effect on ER beta' can be confusing. What's the difference between IOD value and relative ratio? Which one is more reflective of the values? Why presented both?

Response: Thanks for the comments. We have used IOD value to quantify the results of immunohistochemical staining, and relative ratio to quantify the results of western blotting. For ERβ, the results obtained from immunohistochemical staining and western blotting results were inconsistent. We think that relative ratio is more reflective of the values than IOD values. We have presented the two results in the manuscript. However, if you think that it is unnecessary, we can delete one of them.

8. Discussion page 13: please check this sentence - Deficiency of E2 significantly impedes bone loss. (How does deficiency in E2 impedes bone loss?)

Response: Thanks. We have improved the description in the revised version.

Deficiency of E2 significantly promotes bone loss and aggravates uterus atrophy [34, 35]. (Discussion section, Paragraph 5, page 11, line 53).

9. Page 13: Please also check this sentence - The reduction of FSH may contribute to uterus atrophy. Isn't it contradictory to the previous statement: high circulating FSH contributes to endometrial atrophy.
Response: Thanks for the critical review. The sentence ‘High circulating FSH contributes to endometrial atrophy’ is correct statement and we have revised it in the new submission.

The increase of FSH may contribute to uterus atrophy. (Discussion section, Paragraph 5, page 12, line 2).

10. Figure legends need to be revised. There's no A, B, C, D labelled at each group. Only A for histological analysis and B for the bar chart. For figures 3 and 6, there are A for the expression bands, B for ER alpha bar chart and C for ER beta bar chart. Typo in the legends. Supposed to be in uterus but legends say in bone.

Response: Thanks. We have improved figure legends in the revised version according to your suggestion.

11. For histological images, should describe what the arrows are pointing at.

Response: Thanks for your suggestion. The arrows have been added in the revised manuscript, which illustrated the expression and distribution of ERα and ERβ.

12. Revise the references list. A few incomplete citation. Full comments are stated on the pdf manuscript which has been uploaded and submitted together with these comments. *Kindly note that I was only able to view the figures and table available in the original document. There is no figures and table available in the revised document.

Response: Thanks. We have revised the reference list and improved citation.