Title: Chinese herb mix Tiao-Geng-Tang possesses anti-aging and anti-oxidative effect and up-regulates expression of estrogen receptors alpha and beta in ovariectomized rats

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Author's response to reviews: see over
Dear Editor,

Thank you for your encouraging evaluation and we also would like to thank the reviewers for their careful reading. Their comments, critics and suggestions are constructive and helpful for improving our manuscript. We believe to have resolved all the issues raised by the reviewers. The responses are listed in the following pages. However, should there be some misunderstanding and any further requests, we will also be very happy to consider and further revise the manuscript accordingly.

We look forward to hear from you.

Yours sincerely
Xu Lian-wei
Response to comments of the reviewers (the original comments are in bold face)

Referee 1: 7042809515791727_comment

1. They need to show representative expression patterns of protein level.
   Done as suggested in Fig. 2B.

2. ER expression is related with anti-oxidant effect in hypothalamus?? The authors need to show their rationals. Is it casually related or resultantly related with it??
   There are GAP between ER alpha, beta expression and SOD activity... They need to clarify the relationship logically not casually.
   We showed that TG-decoction has anti-oxidative activity and independently found that this decoction led to increased ER expression. These findings are both effects of TG-decoction but are not necessarily directly related to each other. We modified the relevant parts in the discussion section so that this issue is presented more clearly.

3. I don't think they are showing their mechanism clearly in this study. In addition, the discussion section is not enough to suggest their mechanism. They need to upgrade their discussion.
   The reviewer is right that the mechanism of the herb mixture is not shown with only the data obtained in this study. Traditionally, Chinese medicine is based on empirical experience and many concepts (likely Ying and Yang) are completely different from those of western medicine and from those of experiment-based science. We and others in the field of Chinese medicine have just started trying to understand Chinese medicine in a scientific and biological way. This study is only a first step and a pilot study toward understanding the mechanism of this Chinese herb mixture. It is a long way to go and in fact, we are launching further studies to define the precise composition and to identify targets of the herb mixture. We believe these studies will provide us more data. We upgraded our discussion accordingly.

Referee 2: 2409709285867901_comment

Major compulsory Revisions

Introduction
You should explain Why the ERa and ERb expressions were measured in this article. According to your title, this is the most important point in this manuscript.

Response:
One possible cause of diminished responsiveness of the hypothalamic-pituitary-gonadal axis to estradiol during aging has been hypothesized to be a decrease in the
density of estrogen receptors (Wilson et al. 2002). We aimed to examine whether or not our TG-decoction could elevate expression of ERs which may lead to recovering of the diminished responsiveness to estradiol. We added this rational in the introduction section.

Discussion
1. Adams et al. (J. Neurosci. 2002. 22: 3608-14) and many other investigators were suggested that estrogen exposure was down-regulated the ER-α expression in in vitro and in vivo experiments. However, your result was not as same tendency, therefore you must explain this contradiction.

Response:
In the cited article, Adams et al reported reduction of ERα in hippocampus and in a very specific manner in the presynaptic pool. In our study, we examined hypothalamus. In addition, there are also studies reporting increased expression of ERs in hippocampus and hypothalamus upon E2 treatment [Rune GM et al. 2002, Tani H et al. 1997, Zhang YS et al. 2011]. This may explain the contradiction. However, indeed, further studies are necessary to clarify this issue.

2. “The major finding…..body balances.” These sentences were unclear. Why did the up-regulation of ERα and ERß introduce “regulate body balances”? Please explain.

Response:
“body balance” is a major concept of Chinese medicine. However, the reviewer is right that there is no direct evidence of this rather abstractive body condition. We removed this sentence.
As for up-regulation of ERα and ERß, our rational is that this may increase responsiveness to estradiol which is decreased during aging and in menopause. We modified the relevant part in the discussion accordingly.

3. “The mild estrogen-…..quercetin.” Please mention the relative composition of these estrogenic compounds in TG-decoction.

Response:
We specified relative composition as suggested and modified in the text accordingly: The mild estrogen-like direct effect of TG-decoction is reasonable since several constituents are known to contain flavonoid aglycones (kaempferol, apigenin, quercetin, luteolin and breviflavone B) which have estrogen-activity.

4. Please mention the health effect of up-regulation of ERα and ERß in this manuscript.

Response:
Up-regulation of Erα and ERß may increase responsiveness to estradiol which is decreased during aging and more drastically decreased in menopause. However, we do not know if it is the case and need to further investigate the effect of change (increase and decrease) of expression of ERs including effects on various signal pathways.

5. Discussion part is too short.

Response:
After reading the comments and suggestions of the reviewer, we indeed also feel there are more to be discussed and now expanded and reconstructed the discussion section accordingly.

Minor Essential Revisions
1. Please mention the complete word before the first abbreviation word.
Response:
Done as suggested.

2. Please mention the breeding type of rat and production field.
Response:
Added the information at the beginning of “animal and treatment”.

3. Please mention the city and country in the research laboratory, reagents, and instruments.
Response:
Done as suggested.

Referee 3: 3101307415902906_comment

Major Compulsory Revisions
1. Title: In title TG-Decoction is not necessary, deleted from title.
Response:
Changed to “Tiáo-Gêng-Tâng”. Besides TG-decoction, there are also several other Chinese herb mixture used for treating menopause syndromes. Therefore we feel the need to specify which one is meant in this study.

2. List of Abbreviation is provided.
Response:
We have added a list of abbreviation at the end of the manuscript.

3. Abstract:
a) In methods, 6th line (MDA) were measured using standard assays correct as–were measured using Thiobarbituric acid method.
Response:
Modified as suggested.

b) In results: The result should be rewritten by good English to get the clear meaning.
Response:
Done as suggested. The authors asked a native English-speaking scientist (Dr. R. Nguyen of the Department of Pediatrics, University of Maryland, Baltimore) to edit the manuscript.

c) ERa was the correct abbreviation? or ER#-corrected throughout manuscript
Response:
Corrected.

4. Background:
a) In second paragraph the meaning of sentence is not properly presented and rewrite.
Response:
We modified.

b) In third paragraph—fifth line “So far, only……TG Dcoction” – rewrite the sentence again, which presented with poor English.
Response:
We have rewritten the sentence.

5. Methods:
a) Preparation of TG-Dec: All the botanical name of plant should be written in italic and sub-species name should be start with small letter e.g: Radix Paeonie Alba - corrected as Radix paeonie alba,…correct all plants name similarly
Response:
Done as suggested.

b) The complete details of name of Hospital with place should be mentioned.
Response:
We have added the details.

c) In method, whether Tiao-Geng-Tang was commercially available product, then whom you procured details should be given or if you prepared kindly mention the details, when you collected plants, which part of the plant is used and which basis you have selected the dose to administration to animals.
Response:
Tiao-Geng-Tang is not available commercially. However, all the 10 herbs it contains are available from traditional pharmacies in China. A prescription of Tiao-Geng-Tang
is a list of the 10 herbs and amount (g) of each. Patients purchase these herbs in the defined amounts in packages and cock them in approximately 3 fold amount of water. The constituents are extracted in water during cocking and intaken by the patients. We added some description for this procedure and modified the dosage calculation for rats.
We also added a table 1 to describe the details of the herbs including dosages in human.

d) Dose of TG-Decoction not clearly mentioned? What it means 19.37g crude herb/kg.d , justify properly.

Response:
Dose of Chinese herbs for patients is given in dry weight in gram. An adult (60kg body weight) daily dose is 166 dried herbs for the TG-decoction. Dose for rats was calculated as:

\[
166g \text{ (adult daily dose)} \times \frac{0.2kg \text{ (rat weight)}}{60 \text{ kg (adult weight)}} \times \frac{7}{7} \text{ (body-surface ratio rat:human)} = 3.87g/rat
\]
We have modified the description accordingly.

e) Whether acute toxicity study was conducted?

Response:
As for most Chinese herb mixtures, there is no systematic toxicity study for the TG-decoction. However, this decoction has been used for over 30 years and no direct toxicity has been reported. Nevertheless, laboratory studies are indeed necessary to define toxicity of TG-decoction.

f) Correct sentence as: All specimens and samples were stored at -80OC until used for assay.

Response:
Modified as suggested.

g) In Laboratory measurement section: FSH,LH, E2 … sentence not giving proper meaning –rewrite and give complete information of institution mentioned with place in brackets

Response:
We have modified and put the reagent institution in brackets.

h) SOD, Total antioxidant capacity and MDA assay procedure should be give briefly.

Response:
These procedures are established ones. We added brief description of principles.

i) In section Ultrastructure of hypothalamus mitochondria – The complete
Minor Essential Revisions

1. Statistical analysis: In this section “Stastic analysis –should be corrected as stastical analysis was carried….. and One way ANOVA –corrected as One way analysis of Variants (One-ANOVA).

Response:
Modified according to the advice.

2. Discussion: Discussion which is not mentioned not sufficient to justify, still required to add some more information.

Response:
After reading the comments and suggestions of the reviewer, we indeed also feel there are more to be discussed and now expanded and reconstructed accordingly.

3. Typographical errors, grammatical errors, stylistic suggestions etc throughout manuscript should be addressed.

Response:
Done as suggested.

Referee 4: 4722198666076807_comment

Major Compulsory Revisions:

1) Discussion about the advantage of multiherbal formulation & decoction over individual herb known to have antioxidant, mild estrogenic activity because of presence of specific phytoconstituent would be more appropriate.

Response:
We modified the discussion accordingly.

2) Some of the parameters show lots of std. deviation, are they still statistically significant?

Response:
Data are statistically analysed and only those which had p<0.05 were given as significant.

3) Results in Figure & text should match.
Response:
We check carefully again and corrected mismatches.

4) Discussion needs to be completely rewritten.
Response:
Revised.

5) Importance of expression of ER # & ER # should be discussed in terms of side effects/ adverse effects of estrogen.
Response:
We hypothesize that increased expression of ERs may increase responsiveness to estrodial. However, we do not yet know if this is the case. Also side effects need to be explored in further studies. Future studies will hopefully shed light on effect of increased expression of ERs including their effects on signal pathways.

Minor Essential Revisions:

1) In the section Animals and treatment, paragraph 2, explain the calculations in detail. How much is the human dose, & the conversion of gms of crude herb to dose in ml?
Response:
We have added table 1 to describe the details of the herbs including dose in human and calculated dose for each rat as following:

\[
166\text{g (adult daily dose)}\times\frac{0.2\text{kg (rat weight)}}{60\text{ kg (adult weight)}}\times\frac{7}{7\text{(body-surface ratio rat:human)}} = 3.87\text{g}
\]

2) In the section of results, expression of ER # & ER #, always use statistical terms not slightly. Alos here results in text & fig does not tally.
Response:
We checked again and corrected mismatches.

3) Minor spelling & grammatical mistakes.
Response:
Checked.

4) In the section discussion, paragraph 1: last sentence of paragraph 1 what is milder & slow effect? Explain you got better effects with TG for the same duration of treatment than premarine, explain?
Response:
Generally, effect of Chinese medicine is slower and milder than western medicines which are mostly synthetic chemicals or highly dosed extracts. We do not have data
suggesting that TG-decoction has better effects than Premarin. Our data only suggest that the effects are difference in the rat-model. As why the effects are different and what is the cause and consequences of these differences, we do not have answer at the present stage. The present study is just the first step toward scientific understanding the complex mechanism of TG-decoction in regulating hormone balance which raises more questions than delivers answers. It is a long way to go and in fact, we and others are initiating further studies including defining the precise composition and to identifying targets of the herb mixture.

Editorial requests

Please also document in the Methods section of the revised version of the manuscript the details of the institutional review board that granted ethical approval for the animal experiments.

Response:
We have added the information at the beginning of “animal and treatment”.