Author's response to reviews

Title: Evaluating the antidiabetic effects of Chinese herbal medicine: Xiao-Ke-An in 3T3-L1 cells and KKAy mice using both conventional and holistic omics approaches

Authors:

Zhenzhong Yang (paulyzz@126.com)
Feng Zhang (1194299708@qq.com)
Zheng Li (lizheng1@gmail.com)

Version: 2 Date: 10 June 2015

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

The authors would like to thank the reviewers for their very constructive suggestions. We have addressed the reviewer’s comments fully one by one following each comment in the following section. We believe the revision has addressed reviewer’s comments and greatly helped us improving the manuscript.

**Point-by-point response to reviewers’ comments**

**Reviewer #1**

**Q1:** It was better that author explained about the 3T3-L1 cells and KKAy mice in Background section.

**Response:** We thank the reviewer for this suggestion. The 3T3-L1 cell is a well characterized cell line for investigating adipocyte differentiation and lipid accumulation, and KKAy mice is an excellent animal model of T2D, characterized by obesity, hyperglycaemia, dyslipidaemia, and insulin resistance. This description has been added to Background section of the revised manuscript and highlighted in yellow.

**Q2:** Are there similar to previous work in this area in order to compare your work with theirs?

**Response:** In previous studies, Nan et al have reported that XKA could alleviate hyperglycemia and hyperlipemia in diabetic patients and attenuate the blood glucose levels in rats, mice and rabbits (Nan Z, Nan H, He Z, Nan J, Liu D: Treatment type 2 diabetes with Xiao-Ke-An, 920 cases of clinical study and experimental animal research. Academic Periodical of Changchun College of Traditional Chinese Medicine 2005, 21(1):13-15.). To elucidate the effective ingredients and their targets as well as the mechanisms of XKA, our group have investigated the therapeutic mechanisms of XKA in the treatment of T2D in mice using a Fangjiomics approach (Yang Z, Liu W, Zhang F, Li Z, Cheng Y: Deciphering the therapeutic mechanisms of Xiao-Ke-An in treatment of type 2 diabetes in mice by a Fangjiomics approach. Acta Pharmacologica Sinica 2015, 36(6):699-707.). However, a more comprehensive evaluation of the
pharmacological effects of XKA in T2D is needed for better understanding of its therapeutic effectiveness and action mechanisms. In this study, XKA was investigated for its therapeutic effects in 3T3-L1 cells and KKAy mice with both conventional and holistic omics approaches. The results were mainly consistent with that of the previous studies, and also provided both phenotypic evidence and underlying action mechanisms for the clinical use of XKA treating T2D.
**Reviewer #2**

Q1: XKA are composed of eight herbal medicines of variety types, therefore a conclusive statement cannot be drawn. Hence, the author should clearly indicate what are the eight herbal medicine used in XKA? The percentage of each components, dosages and mixture methods of the eight compositions in XKA should be carefully described. It is also important to include the method of standardization for XKA mixture used in this study.

**Response:** Thank you for the suggestion. XKA consists of *Rehmanniae radix*, *Anemarrhenae rhizoma*, *Coptidis rhizoma*, *Lycii cortex*, *Lycii fructus*, *Polygonati odorati rhizoma*, *Ginseng radix et rhizoma* and *Salviae miltiorrhizae radix et rhizome* with a 6:5:2:4:2:3:2:3 weight ratio of the eight herbal compositions. It was manufactured by Jilin Tonghua Huaxia Pharmaceutical Co. Ltd. (Jilin, China). Briefly, the *Ginseng radix et rhizome* and half of the *Anemarrhenae rhizome* were crushed into powder. *Coptidis rhizome* was crushed into powder, which was further extracted with water and filtered. The remaining half of the *Anemarrhenae rhizome* and the five other herbal medicines were extracted with water together and filtered. These two filtrates were concentrated together to syrup. Finally the powder was mixed to the syrup and dried. This has been added to **Methods** section of the revised manuscript and highlighted in yellow.

Q2: The authors should discuss what are the possible compounds in XKA that contribute to the anti-diabetic activity.

**Response:** Thank you for the suggestion. In our recent article (Yang Z, Liu W, Zhang F, Li Z, Cheng Y: Deciphering the therapeutic mechanisms of Xiao-Ke-An in treatment of type 2 diabetes in mice by a Fangjiomics approach. Acta Pharmacologica Sinica 2015. 36(6):699-707), the possible active compounds that contribute to the anti-diabetic activity of XKA were investigated. Phenolic acids derived from *Salviae miltiorrhizae radix et rhizoma*, xanthones derived from *Anemarrhenae rhizoma*, iridoids derived from *Rehmanniae radix*, triterpenoid saponins from *Ginseng radix et rhizoma* and alkaloids from *Coptidis rhizoma* might be the principal anti-diabetic
Q3: XKA leads to inhibition of adipocyte differentiation at high concentrations of 250 and 500 µg/ml. Please provide a clearer picture (bright field) to show the adipocyte differentiation. Please indicate what is the solvent used to dissolve XKA for the in vitro assay. The experiments should be repeated with this solvent as negative control.

Response: Thank you for the suggestion. The extracts of XKA were dissolved in DMSO with the final concentration less than 0.1% for the in vitro experimental study, and the same vehicle was used in the control group. Part of Fig 1A (Fig 3 in the manuscript) was enlarged at the right of Fig 1. The orange drop in Fig 1 was the lipid droplet stained by the Oil Red O. This was the representative pictures of preadipocyte (A) and differentiation induced cells with Oil Red O staining at XKA concentrations of 0 (B), 500 (C), 250 (D), 125 (E) and 62.5 (F) µg/mL. Therefore, it indicated that XKA could reduce lipid accumulation in 3T3-L1 cells in a dose-dependent manner.

Figure 1. Effect of XKA on adipocyte differentiation.

Q4: Please show whether the XKA treatment affects insulin level in diabetic mice.
Response: The reviewer raised a really good point. We measured the insulin level with the serum sample left from the experiment. As shown in Figure 2, XKA did not significantly affect insulin level in diabetic mice. However due to the limited serum volume, some of the mice didn’t get enough serum left for the serum insulin level measurement. This measurement was carried out for the mice whose serum was sufficient. The tested sample number of each group was uneven, varying from 6 to 10. Therefore, this figure was not added to the manuscript.

![Figure 2. Effect of XKA on serum insulin level.](image)

Q5: Microarray. It is unclear that for the microarray data, what is the concentration of the drug used in the treatment group and what is the tissue samples that were collected for RNA isolation?

Response: We apologize for the confusion. The livers from non-diabetic controls, diabetic models and XKA (1.5 g/kg) treatment groups were stored in liquid nitrogen for further microarray experiment. This was described in the Animals and treatment section of the revised manuscript.

Q6: Please show the values ±SD in the bar charts for Figure 5(A&B), Figure 6 (A&B) and Figure 7.

Response: The means ±SD in Figure 5(A&B), Figure 6 (A&B) and Figure 7 was
shown in the revised manuscript. They were also presented below.

**Figure 3.** *In vivo* glucose levels down-regulation effect of XKA in KKAY mice.

Fasting (A) and non-fasting (B) blood glucose was determined at indicated time. All values are means ± SD (n = 10-11). *p < 0.05 vs. KKAY mice, **p < 0.01 vs. KKAY mice.

**Figure 4.** Effects of XKA on oral glucose tolerance test (OGTT). OGTT was carried out at day 28 (A), and the AUC of OGTT was calculated (B). All values are means ± SD (n = 10-11). *p < 0.05 vs. KKAY mice, **p < 0.01 vs. KKAY mice.
Figure 5. Effects of XKA on body weight in KKAy mice. All values are means ± SD (n = 10-11).

Q7: Figure 8 (network). Figure is not clearly seen. More comprehensive descriptions should be provided to explain the network.
Response: This figure was used to illustrate the reverse rate values of the nodes in the network, and then the holistic reversal effect of XKA could be seen intuitively in the T2D network. The color of the nodes in the network presented the reverse rate of the nodes with the treatment of XKA. Because of the large number of nodes and connections in the network, it was not easy to get all the details presented clearly. However, thanks to the high resolution, the details of the figure could be seen after being enlarged. More descriptions about this figure has been added in the revised manuscript.

Q8: Figure 9 (Top 10 upregulated genes) shows the top 10 upregulated genes in diabetic model and drug treatment groups. Please indicate what does drug treatment means (high or low dosage?). There is no descriptions in the text regarding Figure 9, so the dose, comparison made and other experimental details are unknown.

For most of the genes, we can detect that there are no differences among diabetic model and drug treatment. For some genes, decrease level are shown in drug treatment group. Error bar and statistical significance of the RS differences should be shown from (n=5), so that the effect of the XKA drug can be judged accordingly.
Authors should verify the microarray data by qPCR or Western blotting, especially those upregulated genes mentioned in Figure 9.

Response: Thank you for the suggestion. The livers from non-diabetic controls, diabetic models and XKA (1.5 g/kg) treatment groups were used for microarray experiment. After t-test analysis, we found that there were not statistical differences between diabetic model and XKA (1.5 g/kg) treatment group for several of the top 10 upregulated genes. Therefore, Figure 9 was removed in the revised manuscript.

The microarray data was verified by real-time quantitative RT-PCR. Some representative results were shown in Figure 6 (Fig. 9 in the revised manuscript).

![Figure 6](image)

Figure 6. Validation of microarray gene expression data by real-time quantitative RT-PCR. Black bars indicate the relative gene expression from microarray data, while gray bars indicate that from real-time quantitative RT-PCR. (A) Gpd2, (B) Fgf1, (C) Gnai1. All values are means ± SD (n = 5). # $p < 0.05$ vs. C57BL/6J mice, ## $p < 0.01$ vs. C57BL/6J mice, * $p < 0.05$ vs. KKAy mice, ** $p < 0.01$ vs. KKAy mice.

Please do not hesitate to contact me if you need any further information and clarification regarding this manuscript. The authors appreciate very much the editors and the reviewers for reviewing this paper and providing the precious feedback. I look forward to hearing from you on further decision on this manuscript.

Best regards,

Zheng Li

Tianjin University of Traditional Chinese Medicine, Tianjin, China

Email: lizheng1@gmail.com