Author’s response to reviews

Title: Alcohol extract from Vernonia anthelmintica willd (L.) seed counteracts stress-induced murine hair follicle growth inhibition

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Author’s response to reviews:

Responses to reviewers

Dear Editor and Reviewers,

Thank you for your letter and the reviewers’ comments concerning our manuscript entitled “Alcohol extract from Vernonia anthelmintica willd (L.) seed counteracts stress-induced murine hair follicle growth inhibition” (BCAM-D-17-01521R1). We appreciate for your warm work earnestly. The comments are all valuable and helpful for revising and improving our work. According to the reviewers’ constructive suggestions, we have modified the whole manuscript carefully and improved our language by a native English speaker. We hope the language is now acceptable for the next review process. Revised portion are marked in the paper.

Once again, thank you very much for your positive and constructive comments and suggestions.

We are looking forward to your information about our revised paper.

Your sincerely,

Prof. Jing Shang
The detailed corrections in the paper and responses to the editor/reviewers’ comments are listed point by point as following:

Responses to Reviewers’ comments:

Reviewer #1 (Mayuree Kanlayavattanakul): Language corrections is need before being published

Answer:

Thank you for your kindly suggestion. We tried our best to improve our language and revise our whole manuscript with the kindly help of a native English speaker: Praveen Kumar Kalavagunta (postdoctor, major in pharmacology) from the State Key Laboratory of Natural Medicines, China Pharmaceutical University. He reviewed and embellished the manuscript.

We hope the language is now acceptable for the next review process.

In addition, a copy of our manuscript showing our changes by either highlighting them or using track changes was uploaded as a supporting information.

Reviewer #2 (Feng-Lin Yen): The manuscript is very interesting. Authors revealed that AVE can attenuate the stress-induced hair follicle growth inhibition in C57BL/6 mice in vivo and in vitro, and may be used as an additive in hair growth product. Authors should be improved the resolution of all figures before publication.

Answer:

Thank you for your kindly suggestion. We tried our best to improve our language and revise our whole manuscript with the kindly help of a native English speaker: Praveen Kumar Kalavagunta (postdoctor, major in pharmacology) from the State Key Laboratory of Natural Medicines, China Pharmaceutical University. He reviewed and embellished the manuscript.

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Reviewer #3:
Reviewer 3

1. Please, have the manuscript carefully checked by an native english speaker (see "so on" line 5 background, last line M&M "anagen Induction" subchapter)

   Answer:
   Thank you for your kindly suggestion. We tried our best to improve our language and revise our whole manuscript with the kindly help of a native English speaker: Praveen Kumar Kalavagunta (postdoctor, major in pharmacology) from the State Key Laboratory of Natural Medicines, China Pharmaceutical University. He reviewed and embellished the manuscript.
   We hope the language is now acceptable for the next review process.
   In addition, a copy of our manuscript showing our changes by either highlighting them or using track changes was uploaded as a supporting information.

2. Add in the abstract that AVE has been already shown to stimulate hair growth (this is a very important background info)

   Answer:
   Thank you for the constructive suggestion. We quite appreciate your insightful comment. We added the sentence “Its alcohol extract (AVE) has been proved to promote hair follicle growth in C57BL/6 mice.” (Line 32, page 2) according to the reviewer’s request in our revised manuscript.

3. Explain the rationale why the study was performed in the abstract and introduction (why would AVE have an effect on the release of neuropeptides?)

   Answer:
   Thank you for the kindly suggestion, we are very sorry for our unclear introduction and added more detailed explanations in our abstract and background of revised manuscript in accordance with the reviewer’s concern.
In the Background, we illustrated that stress may act as a precipitating factor in the onset or exacerbation of hair loss. Numerous of studies have proved that hair growth is profoundly influenced by neuromediators involving in systemic stress responses. Hair-cycle-dependent fluctuations in cutaneous innervation may reflect increased expression for neuropeptides. It is suggested that hair follicles are source and target of neuropeptides, indicating an intimate interaction of nerve fibers and their cutaneous target organs[1-3].

Furthermore, in the previous study of our research group, we investigated the effects of AVE and fluoxetine on chronic restraint stress and chronic unpredictable mild stress model in mice, and our results showed AVE can attenuated the depressive situation in C57BL/6 mice. However, these data and results had not been published. We are still performing the experiments and investigating the possible mechanism.

Considered the important role of neuropeptides in regulating hair follicle growth and hair cycle, we evaluated the effect of AVE on the release of neuropeptides.

4. Background: do not generalize hair loss and specify for which hair loss AVE would be promising (i.e. stress seems to be involved in alopecia areata, telogen effluvium, etc. but not in androgenetic alopecia)

Answer:

We are very sorry for our unclear introduction in this section. As the references reported, alopecia areata (AA), telogen effluvium, lichen planopilaris (LPP) may have relations with stressful events[4-6]. Thus, we added more information about stress-induced hair loss in the background of our revised manuscript (Line71-72, page 4).

5. The sentences regarding the central nervous system is not really relevant to make the link with the aim of the study. Please, revise adding information on stressed-mediated hair loos (explaining the role of SP and CGRP), how AVE promotes hair growth, and what other property of AVE suggests that it could counteract stressed-induced hair loss.

Answer:

We agree the reviewer's good advice, and we deleted the sentences regarding nervous system and revised the whole background in order to make our point more clear and closer to the aim of our study.
Substance P (SP) and calcitonin gene-related peptide (CGRP) have been reported to effectively manipulate skin and immune cell functions such as cell proliferation, cytokine production, and antigen presentation under normal and pathological conditions in C57B/6 mice model[7-9]. In addition, in the absence of a functional perifollicular innervation, both two neuropeptides have similar inhibited effects on organ-cultured hair follicle growth[8].

We added more information regarding stress-mediated hair loss and the role of SP and CGRP according to the reviewer’s request (Line100-105, page 5).

6. Line 23, the sentence starting with ""In C57BL/6"", needs to be corrected as in all systems (human, mice, rat) melanogenesis is coupled with only anagen, and it is off during catagen and telogen.

Answer:

We are sorry for our inappropriate expression. Thank you for your kindly suggestion. Therefore, we modified the sentence starting with “In C57BL/6 mice…” to “In all mammalian species, follicular melanogenesis is coupled only during anagen stage of the hair cycle, ceases during catagen and absent through telogen.” (Line 82-83, page 4)

7. Please, explain better why C57BL/6 is the optimal model, I do not buy what you have written, human is the ultimately good model. However, I would buy it if you write and quote that many study using this model focused on understanding the neuro-endocrine regulation of hair cycle, and that were used to test other compounds.

Answer:

There are several reasons why C57BL/6 mice is the ideal model for the study of hair loss research.

1) The synchronous nature of hair follicles in C57BL/6 mice, either spontaneous or induced by depilation, not only provides an opportunity to study large numbers of hair follicle in the same phase of the hair growth, but the intensely pigmented hair facilitates easy staging of anagen (gray to black skin) and telogen (pink skin)[10, 11].

2) There are mountains of studies from research groups of P.C. Arck and Ralf Paus who used C57BL/6 mice as a tool to investigate the hair follicle biology and hair growth changes after stress stimuli. The criteria for the recognition of distinct hair cycle stages presented by Ralf Paus are highly reproducible and reliable in all C57BL/6 mice over a very wide age range[7, 12-14].
3) Hair cycle-related plasticity in the sympathetic innervation of skin and hair follicle in C57BL/6 mice has been demonstrated in previous studies. The bi-directional interactions between the hair follicle and its innervation play a part in hair growth control. For experimental evidence, stress exposure alters the number of immunohistochemically detectable SP+ and/or CGRP+ sensory nerve fibers in the dermis[8].

4) Liu et al. found up-regulation of SP protein expression in cutaneous peripheral nerve fibers in chronic restraint stress mice. In addition, SP receptor antagonist (RP67580) significantly ameliorated stress-induced alterations in hair follicle score and induced increase of SP+ nerve fibers number[15].

We appreciate very much for your constructive suggestion and we realized that our description about C57BL/6 mouse as an ideal animal model for hair growth research was too simple and not persuaded. Therefore, we added more detailed description in Background of our revised manuscript.

In addition, the related references were added in the revised manuscript.

8. Line 12, page 5, you are generalizing the FDA approved drug, minoxidil and finasteride are not given to for all hair loss, please specify what it is relevant in the context of your study.

Answer:

We appreciate your kindly advice. It is necessary to demonstrate the background of these two FDA-approved drugs. Minoxidil is used in the treatment in alopecia areata, chemotherapy-induced alopecia, hair transplant, and finasteride is efficacious for androgenetic alopecia[16, 17]. We added relevant information in our revised manuscript. (Line 113-115, page 6)

9. I am not aware with any adverse effect of minoxidil. Please, find supporting references.

Answer:

As the references reported, adverse effects of minoxidil include contact dermatitis, facial hypertrichosis (because of local/systemic absorption or possible contamination of them), and transient increasing hair shedding in the first month of use (caused by the encouragement of hairs already in the telogen phase to shed early)[18-21]. We added the related references in our revised manuscript.
10. M&M: It is missing the number of mice used for the study.

Answer:

We are very sorry for the deficiency of data. We used forty C57BL/6 mice for this study, the animals were randomly assigned to four subgroups (n=10 each group).

We added this data in the Material and Method of our revised manuscript. (Line 172, page 8)

11. This is a very major issue: you did not use the correct criteria, despite of the fact that you quoted the correct guide, for the hair cycle analysis. Morphology of dermal appilla and sebaceous gland has nothing to do with hair cycle. Instead, morphology of the hair bulb, how deep is the hair bulb, number of proliferating hair matrix keratinocyte below Aubers line, etc. are the important criteria. Given this important issue, hair cycle should be re-analize following the instruction of the guide.

Answer:

We very appreciate your good advice. We read once again the comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis[22, 23].

Therefore, we re-analyzed the change of murine hair follicle growth and hair cycle among different groups. With reference to related hair growth research, we added more criteria in the results of our revised manuscript according to the reviewer’s request. Hair parameters (distance between hair germ and subcutaneous layer, thickness of dermis) were measured from representative areas at a fixed magnification of 100×[24].

12. Although the colour of the skin is a very good indicator of hair cycle stage, it cannot be use as the only criteria for the hair cycle analysis.

Answer:

We agree with your good advice, and added more hair parameters concerning the reviewer’s request in previous 11# comment in our revised manuscript.

We assessed the hair follicle growth and hair cycle among four groups via both qualitative and quantitative criteria.
As for the qualitative criteria:

1) color change of dorsal skin in C57BL/6 mice
2) morphological changes of hair follicles

As for the quantitative criteria:

1) total number of hair follicles
2) hair cycle score
3) the length of hair germ and subcutaneous layers
4) thickness of dermis

We thank you again for this constructive suggestion, it makes our research and article more professional and rigorous.

13. The term ""degenerative morphological changes"" does not exist. What do mean with this: catagen promotion or hair follicle cytotoxicity?

Answer:

It is true as the reviewer pointed out that our expression is inaccurate.

According to the time-scale for the hair cycle of C57BL/6 mice in the comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis, we evaluated the morphogenesis of hair follicles among four groups[7, 14].

Compared to the CTRL group, hair follicles in stressed group showed narrower bulb, decreased number of melanin granules in the precortex region and onion-shaped dermal papilla. Based on the criteria described in the references, most hair follicles in the RS group entered into catagen II / III on day 18 after depilation. However, hair follicles in the CTRL, AR and MR group were still in anagen VI. This result suggested CRS inhibit hair growth by promoting catagen.

In the revised version, we deleted the term “degenerative morphological changes” and modified our description of results (Line 279-290, page 14).
14. It is important to quantify the number of Ki-67+ hair matrix keratinocytes below the Aubers line as further confirmation of the hair cycle result.

Answer:

We are very sorry for our negligence and the deficiency of data. With the references of previous studies of hair growth, we only performed the qualitative analyze of Ki67+ hair matrix keratinocytes[25].

We understand that the quantitative evaluation of Ki67+ hair matrix keratinocytes may better reveal and confirm the result of hair cycle change in different groups. However, we did the immunofluorescence histochemistry by using cryosections to detect the expression of Ki67+ keratinocytes located in the hair matrix and we have run out of the animal sample. We realized the quantitative analyze of Ki67+ hair matrix keratinocytes could be an important criterion for evaluating the hair cycle and follicle growth. Therefore, in our next article and study we will perform both the qualitative and quantitative evaluation of Ki67+ keratinocytes to make our results more persuasive. Thank you so much for the constructive suggestion.

15. K19 immunostaining is totally unspecific in the inner root sheath. I am not sure it is detecting K19+ cells in the bulge. Please, show higher magnification of bulge outer root sheath, and use other markers better suited for detecting bulge stem cells, i.e. K15 and CD34. Yet, it is important to describe why did you evaluate this marker because it is not really relevant for anagen prolongation but more for anagen initiation, i.e. telogen to anagen transition.

Answer:

K19 is a biomarker for hair follicle stem cells. These cells are capable of forming the follicle, epidermal and sebaceous gland, which play a significant role during hair follicle growth and regeneration. Thus, we detected the K19+ stem cells to investigate whether AVE could contribute to the hair regrowth function.

We are very sorry that we did not show higher magnification. Before we did the experiment, we referred several studies about K19+ expression in hair bulge region, they detected K19+ in the bulge of hair follicles at the scale bar of 100μm[26, 27].

In our further study, we will take higher magnification of the bulge area of outer root sheath to make our results more persuasive. In addition, we will try to examine other important markers of stem cells (K15+, CD34+) concerning the reviewer’s good advice.
16. Also for SP and CGRP the images are not convincing. You are indicating as positive staining the unspecific staining on the arrector pili muscle. Show higher magnification, better quality picture. To be sure that what you are detecting are indeed nerve fibers, you should use also PGP9.5 as marker.

Answer:

We thank the reviewer for the constructive suggestion.

As the reference convinced, single nerve branches innervate the arrector pili muscles at the peripheries of hair follicles and appear in the dermis[12, 14, 28]. In Fig.5a of our article, we detected the SP+ and CGRP+ nerve fibers not only located around hair follicle but also distributed in the dermis of skin. The white square frame showed magnification of nerve fibers.

PGP 9.5 is a marker of nerve fibers document the hair cycle-associated plasticity of follicular, and it is an excellent marker for identifying the change of murine hair cycle. However, in the current study, we focused on the SP+ and CGRP+ nerve fibers and the possible mechanism neuropeptides regulating the hair growth. Thank you for your kindly advice, and we have already chosen PGP 9.5 as a marker to detect hair cycle changes in the experiment we are performing now. The effects of CRS and each individual component of AVE on PGP 9.5 nerve fibers in C57BL/6 mice will be shown in our next article.

17. I am also not convinced about the down-regulation of NK-1R expression, please confirm this with immunohistology. "

Answer:

We are appreciative of the reviewer’s suggestion, but we are very sorry that we have finished using all the animal samples. Thus, we seek for the reviewer’s tolerance and understanding that we fail to provide related data.

This article was the first and initial step of our research group to explore the effects of AVE on hair follicle growth. There is no doubt that the limitations of this study still need further investigations and improvements. As for the important role of substance P and its receptor NK-1R participating in the regulation of hair follicle growth, we are now performing the related research. Additional experiments about the expression of NK-1R in hair follicle of C57BL/6 mice after stress exposure will be necessary to investigate in our next article.

Finally, once again, we thank you very much for your good comments and suggestions. It makes us realize that there are still limitations about our work and we will try our best to improve the design of experiments, descriptions of the results and further direction of our research.
References


