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OPEN PEER REVIEW

The Expression of Melanin Concentrating Hormone and Corticotrophin Releasing Hormone Genes in a Stress Model Rats Receiving Formononetin

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About this report

This open peer review (OPR) report shows the public comments made on this article during peer review, by the reviewers, the associate editor and the editor-in-chief, for every revision round, together with the authors' replies. Comments appear only with the consent of the people who wrote them. Reviewers who chose to sign their report are named; the others are shown as "Referee". Comments are edited for language only, and confidential remarks to the editors are not included.

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1 Original submission

2 comments · 19 Jul 2024 – 31 Jul 2024

Referee 1 Reviewer

MAJOR REVISION 19 Jul 2024

1. The novelty of the study was not noted.
2. The aim of the study was not mentioned in the background.
3. The manuscript is not well structured.
4. The kits used are not included.
5. There are no keywords.
6. Inappropriate methodology is seen. How were the 20—and 40-dose doses of formononetin calculated? What were the results, differences, and interpretations of using these doses?
7. The results are very brief and uninformative. The 4 groups of rats should be explained, and procedures and results clarified.
8. The figures weren't addressed in the text.
9. References are not in an appropriate format.
10. The text should be revised using Grammarly.

Associate Editor

MAJOR REVISION 31 Jul 2024

Dear author,
Thank you for submitting your valuable manuscript to the journal. We would like to inform you that the peer review of your manuscript is now complete and that, despite several useful points, your manuscript needs "Major Revision". Below you may find the peer review comments. Please reply to them one by one and submit the manuscript again.
Final Reviewers' Comments:

Reviewer 1:
1. The novelty of the study was not noted.
2. The aim of the study was not mentioned in the background.
3. The manuscript is not well structured.
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8. The figures weren't addressed in the text.
9. References are not in an appropriate format.
10. The text should be revised using Grammarly.

Reviewer2:

1. The term "formononetin" should be consistently spelled correctly throughout the text. Occasionally, it appears as "fornononetin."
2. The expression "The present study focus to find the formononetin impacts..." should be revised for grammatical accuracy, possibly to "The present study focuses on the impacts of formononetin..."
3. Please strengthen the discussion by comparing findings with existing literature. Highlight how the results

align or differ from previous studies, and discuss potential reasons for these similarities or differences.
4. Suggest areas for future research, such as exploring the effects of different doses of formononetin, using different stress models, or examining other related genes or proteins.
5. The conclusion should summarize the main findings, their implications, and the overall contribution of the study to the field. Avoid introducing new information in the conclusion that has not been covered in the main text.

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Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear editor in- chief

By special thanks of valuable comments of dear reviewer to improve the manuscript, the responses were highlighted in the text. Also they were mentioned in the present file.

Response to Reviewer1:

1. The novelty of the study was not noted.

Current chemical analgesic medicines have significant adverse effects, including gastrointestinal issues, emesis, tolerance, or addiction. Therefore, to relief of stress, new medicines derived from plants, as a complementary or alternative approach, are needed to reduce adverse effects. Formononetin is a phytostrogen derivative of *Red clover* which is consumed by to relief stress and hot flash based on traditional medicine. Formononetin exhibits significant estrogenic capabilities, antioxidant and anti-inflammatory properties and previous studies documented its anxiolytic effects based on behavioral tests. However, the molecular mechanisms mediating the anxiolytic effects of formononetin are still unclear. So novelty of the present study may be finding the central mechanisms through the formononetin may mediate anti-stress effects.

2. The aim of the study was not mentioned in the background.

Based on previous studies, formononetin has shown to improve depression via up- regulation of the glucocorticoid receptor, brain derived neurotrophic factor, and promotion of hippocampus neurogenesis. To find some molecular mechanisms related to the anti-stress effects of formononetin, in the present study the impact of formononetin was investigated on the hypothalamic *Mch* and *Crh* gene expression in a rat model of stress.

3. The manuscript is not well structured.

According to the reviewer's suggestion, the manuscript was structurally reviewed.

4. The used Kits are not included.

Based on dear reviewer guidance, the used kits were added to text as following:

Trizol kit (Qiagen, Germany)

cDNA kit (Biotech rabbit, Germany).

SYBR Green I Kit (Takara Bio Inc., Japan).

5. There is not any keywords.

Based on dear reviewer guidance, Key words were added to the text as following:

stress; Mch; Crh; formononetin.

6. Inappropriate methodology is seen. How were the 20—and 40-dose doses of formononetin calculated? What were the results, differences, and interpretations of using these doses?

To select the effective doses of drug, we used behavioual tests related to stress responses such as open field, forced swimming test. So, at first rats in several groups received different doses of the drug and between the used doses, based on behavioural responses we choose 20 and 40 micrograms formononetin to investigate the molecular mechanisms may mediate the anti-stress effects of formononetin

7. The results are very brief and uninformative. The 4 groups of rats should be explained, and procedures and results clarified.

According to the opinion of the respected reviewer, the results were explained with four groups in the text as following:

The *Mch* mRNA level was augmented in the stressed rats (Group II) remarkably compared to the control (Group I) (Figure1. $p \leq 0.05$). *Mch* mRNA level in the rats receiving 20 (Group III) or 40 (Group IV) µg formononetin remarkably decreased compared to the stressed rats (Figure 1. $p \leq 0.05$).

The mRNA level of *Crh* increased significantly in the stressed rats (Group II) compared to that of the control (Group I) ($P \leq 0.05$). Injections of 20 (Group III) or 40 (Group IV) µg formononetin remarkably decreased *Crh* mRNA levels compared to the stressed rats (Figure 2,

8. The figures weren't addressed in the text.

According to the format of the journal, the figures are listed at the end of the text.

9. References are not in an appropriate format.

The references were edited according to the format of the journal.

10. The text should be Grammarly revised.

Dear reviewer, the manuscript has been checked for grammatical problems.

Response to Reviewer2:

1.The term "formononetin" should be consistently spelled correctly throughout the text. Occasionally, it appears as "fornononetin."

According to the suggestion of the respected reviewer, the word formononetin was corrected throughout the text.

2.The expression "The present study focus to find the formononetin impacts..." should be revised for grammatical accuracy, possibly to "The present study focuses on the impacts of formononetin..."

According to the suggestion of the respected reviewer, this item was edited in the text as following:

In the present study the impact of formononetin was investigated on the hypothalamic Mch and Crh gene expression in a rat model of stress.

3.Please strengthen the discussion by comparing findings with existing literature. Highlight how the results align or differ from previous studies.

According to the suggestion of the respected reviewer, the discussion section was edited as following, and it is highlighted in the text of the manuscript.

This study showed that stress increases the expression of the *Crh* gene. This finding is consistent with the previous results that demonstrated the activation of the hypothalamic-pituitary-adrenal (HPA) axis in stressful situations (2, 16). Also, the another study shown that, chronic stress can lead to sustained elevation in the activity of corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus (17).

The *Mch* mRNA levels were up- regulated in the stressed rats. This finding is in agreement with the studies that established the hyperactivity of hypothalamic MCH neurons control stressful behaviors (29, 30). Moreover, the previous study shows that chronic stress activates MCH neural pathways in mice. (31).

4.Suggest areas for future research, such as exploring the effects of different doses of formononetin, using different stress models, or examining other related genes or proteins.

Based on dear reviewer guidance, suggestion for future studies were added in the text as following:

The present study indicated the formononetin may be involve in improving stress by down regulation of hypothalamic *Crh* and *Mch*. However, for precise understanding of the role of formnonetin in management of stress, further studies are needed to investigate the effects of formononetin on other stress related genes or proteins such as orexin, neuropeptide Y, neuropeptide S, phoenixin, calcitonin gene related peptide in acute or chronic stress models.

5.The conclusion should summarize the main findings, their implications, and the overall contribution of the study to the field. Avoid introducing new information in the conclusion that has not been covered in the main text.

Based on dear reviewer guidance, conclusion of the manuscript was edited as following:

The results showed the induction of stress caused a significant increase in mRNA levels of *Crh* and *Mch*. Formononetin exerted inhibitory effects on hypothalamic *Crh* and *Mch* gene expression in the stressed rats. Formononetin may have promising therapeutic properties for anxiety by regulating hypothalamic neural circuits upstream CRH neurons, such as MCH neurons.

2 Revision 1

2 comments · 14 Aug 2024 – 17 Aug 2024

Referee 1 Reviewer

MINOR REVISION 14 Aug 2024

- The novelty and aim of the study were not mentioned in the introduction/background.

Associate Editor

ACCEPT 17 Aug 2024

Dear editor,
I think this manuscript can be accepted.