Author's response to reviews

Title: An-jun-ning, a traditional herbal formula, attenuates spontaneous withdrawal symptoms via modulation of the dopamine system in morphine-dependent rats

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

Thank you for inviting us to submit a revised version of our manuscript entitled “An-jun-ning, a traditional herbal formula, attenuates spontaneous withdrawal symptoms via modulation of the dopamine system in morphine-dependent rats” (MS: 8490900141129929) to be considered for publication in the journal of BMC Complementary and Alternative Medicine.

We appreciate the comments by the reviewers and hereby enclose a point-to-point response to their comments. All changes have been highlighted in the text in red. We hope that you find the revised manuscript acceptable for publication.

Neither the entire paper nor any part of its content has been published or accepted elsewhere. No material submitted as part of a manuscript infringes on existing copyrights, or the rights of a third party. All authors have read and agreed to its content.

Thank you!

Sincerely,

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Replies to the reviewers comments:

Reviewer 1:

Reviewer's report:

The present work describes the effects of An-jun-ning (AJN), a commercial traditional Chinese medicine on the dopaminergic system in morphine-dependent rats. AJN reduced the wet dog shakes or the episodes of writhing induced by morphine and post-treatment with AJN restored the levels of TH, DAT and D2R in the striatum. The authors conclude that the mechanism of the effects of AJN on withdrawal signs may be related to the modulation of the “dopamine” system by AJN. In general the study appears to address an interesting issue. However, I have some major concerns with the study and its interpretations as it stands.

Major concerns:

1) The crucial problem with this paper is the author’s attempt to examine without clear criteria, 2 schemes of AJN, pre-treatment with one dose and post-treatment using 3 doses and different duration of treatment, on morphine-induced signs of abstinence in rats. Besides, the authors rely on dosages of AJN converted from those used clinically, on behavioral signs dependent on locomotor performance of rats without an adequate control response of the treatment on this matter.

   Thanks for the reviewer comments. We designed the two schemes of AJN to evaluate its therapeutic effect by post-treatment and confirm the possible mechanism of dopaminergic modulation by pre-treatment.

   The post-treatment of AJN was scheduled to examine its therapeutic effects on protracted withdrawal symptoms and its ability to interdict the morphine-induced decreases in DAT, D2R and TH, and thus the involvement of DA system in the efficacy of AJN. Usually, a drug is evaluated with three or more doses [1-3]. We chose the minimum of three doses for post-treatment to examine whether there was a dose-dependent relationship in the therapeutic effects of AJN. Previous studies reported the duration of 10 days’ treatment with AJN as an effective therapeutic
regimen in the clinics [4], therefore we chose the regimen in our treatment design.

Pre-treatment could provide us another perspective on the mechanism of AJN, through examination of its possible inhibition of behavior response and neurobiological disturbance in the nervous system. With this scheme, we investigated whether AJN could interdict morphine-induced behavior response and neurobiological changes, and in this way, to confirm the possible mechanism that AJN works on protracted withdrawal symptoms via modulation of the dopamine system. To avoid unnecessary animal use in consideration of the international guidelines for care and use of laboratory animals, we set only one dosage for pre-treatment in our experiments. Additionally, the pretreatment was conducted 30min before each dose of morphine, and it’s an 8 days’ duration.

As for the controls, there is a blank group treated only with saline and a model group treated only with morphine. And the behavior test score in the model group was much higher than that in control group. Thus we managed to evaluate the therapeutic effects on withdrawal symptoms.

References:
2) The main argument of the authors for a reduced behavioral response of treatment is based on the assumption that AJN reverted the number of wet dog shakes or episodes of writhing in rats. In my view, one critical issue is that the authors rely on writhing responses showing mean around 1.1 episodes for morphine group and did not bother to discuss the bias of affecting such low level of responses. It is unlikely that the selected “dependence” condition will detect meaningful protective effects of AJN treatments.

The spontaneous withdrawal model was chosen in this study because the withdrawal process of the spontaneous withdrawal model is much closer to that of patients in the clinic than other withdrawal models, for example, naloxone-precipitated withdrawal model [1-2]. As is known, the acute stage of physical symptoms is short in opioid dependence.

We agree that spontaneous withdrawal from chronic morphine treatment induces only mild signs, e.g., wet dog shakes and episodes of writhing [3]. The episodes of writhing in particular are not many. However, the episodes of writhing in the groups with either AJN pre- or post-treatment were still significantly less than those in the morphine group, indicating the effect of AJN on spontaneous withdrawal symptoms.

References:
The authors should include some more details concerning the main constituents of the commercial AJN selected for this study. The omitted information calls for a great deal of caution in interpreting the data obtained here.

More details concerning the main constituents of AJN would be helpful, indeed. But the literatures about other constituents in the prescription of AJN are rare. And since the *Rhizoma Corydalis* proportion in the prescription of AJN, 39.78%, is much more than other constituents such as *Glycyrrhiza uralensis Fisch.* (2.39%), *Houttuynia cordata Thunb.* (11.93%), *Alpinia oxyphylla* (3.98%) and *Arisaema cum bile* (7.87%), we mainly focused on *Rhizoma Corydalis* and its primary active compound l-tetrahydropalmatine (l-THP) in this study.

4) Authors should provide in the text how the behavioral responses were recorded, e.g., is the observer blind to the treatment of the groups, were the experiments video monitored.

We added the corresponding details in the experimental section. Three observers blind to the groups performed the observation and scored independently, and the scores were averaged as the result for each behavior test. The experiment video is really a good idea. But, unfortunately, we didn't record a video of the behavior observation. We will implement this practice in future studies.

Revised manuscript in methods section:

**Behavior observation**

At 10:00 a.m. on days one, five, and ten after the withdrawal of morphine, the animals were placed individually into Plexiglas cages and observed for signs of spontaneous withdrawal. Following a 5 min acclimation in the cages, the number of wet dog shakes and writhing episodes were monitored during a period of 30 min (Zharkovsky et al., 1993). Three observers who were blind to the groups completed the observation and score independently. Scores were averaged for each behavior test.

Minor

5, -Legends, Figs. 1 – 4, please provide the meaning of NS, JTT, also should include more details, e.g., the effects of AJN are pre or post-treatments (Figs3-4).
We have added some information in the legends and rearranged the figures to make them easier to understand. Additionally, NS means control group, while JTT was just a typo error, is changed to AJN.

Revised manuscript in figure legends:

Fig. 1. Effects of AJN on wet dog shakes and episodes of writhing after spontaneous withdrawal. A: AJN pre-treatment effectively holds wet dog shakes at a low level after 1, 5, 10 days of spontaneous withdrawal. B: AJN post-treatment alleviates wet dog shakes in a dose-dependent manner. C: AJN pre-treatment inhibits episodes of writhing after spontaneous withdrawal. D: AJN post-treatment attenuates episodes of writhing. NS: control group; Mor: morphine group. Data are expressed as the means ± SD (* p < 0.05, ** p < 0.01 vs. morphine group; n=10).

Fig. 2. Effect of AJN pre-treatment on DAT by immunohistochemical staining and autoradiography. A: Representative staining pictures and effect of AJN on DAT by immunohistochemical staining. B: Representative autoradiograms and effect of AJN on DAT by autoradiography with [125I]-β-CIT. Results of two methods are accordant with each other. AJN pre-treatment completely blocks morphine-induced DAT expression decrease, and AJN post-treatment effectively restores DAT expression. NS: control group; Mor: morphine group. Data are expressed as the means ± SD (* p < 0.05, ** p < 0.01, vs. morphine group; n=5).

Fig. 3. Effect of AJN on D2R by immunohistochemical staining and autoradiography. A: Representative staining pictures and effect of AJN on D2R by immunohistochemical staining. B: Representative autoradiograms and effect of AJN on D2R by autoradiography with [125I]-IBZM. Morphine administration reduces D2R expression by about 20%. AJN pre-treatment completely inhibits the morphine-induced D2R decrease. And AJN post-treatment alleviate the decrease in D2R expression in a dose-dependent manner. NS: control group; Mor: morphine group. Data are expressed as the means ± SD (* p < 0.05, ** p < 0.01, vs. morphine group; n=5).

Fig. 4. Effect of AJN on TH density by immunohistochemical staining. Representative staining pictures and effect of AJN on TH density by immunohistochemical staining. TH density in the striatum is decreased in the morphine group. Both AJN pre- and post-treatment effectively
attenuates this decrease. NS: control group; Mor: morphine group. Data are expressed as the means ± SD (* p < 0.05, ** p < 0.01, vs. morphine group; n = 5).

6, -Fig. 1, the results depicted in Panel A and B are for wet dog-shakes only in pre-treated groups? Authors should explain why post-treatment of AJN, panel C-D, only for writhing episodes and the convenience to maintain both panels.

We have edited the figures to make them more scientific, and added details in the figure legends. The results depicted in Fig Panel A are for wet dog-shakes only in pre-treated groups, and results in Panel B are for wet dog-shakes only in post-treated groups. Panel C is for writhing episodes in pre-treated groups, and Panel D is for writhing episodes in post-treated groups.

7, -The manuscript should be carefully examined for typos, grammatical and spelling errors.

All the authors have checked the manuscript carefully. Typos, grammatical and spelling errors are corrected. The manuscript has also been re-edited by the language service of the WebShop.

Reviewer 2:
Reviewer's report:
The article describes that a traditional Chinese herbal medicine reduces two parameters of opioid withdrawal syndrome in rats, along with alterations in the dopaminergic system in the brain. The statistical analysis and data evaluation seems to correct and the results are of relevance to those studying traditional Chinese medicine. However, the scientific advance provided by this study is rather limited, since the intervention is constituted of a complex herbal mixture, and therefore, no mechanism of action can be attributed to it. I recommend publication for the potential interest of those studying or applying traditional Chinese medicine, although a clinical study using human subjects would be way more informative than the current one. Before publication, please improve the English language used in the study; correct the
scientific names in Table 1. And provide a better study justification than the one mentioned: "Traditional Chinese medicines (TCM) have long been used to treat opioid addiction because the 19th Century and many prescriptions were revealed to have ideal therapeutic effects".

We agree that clear mechanism(s) of action can hardly be attributed to a prescription constituted of a complex herbal mixture. On the other hand, traditional Chinese medicines (TCMs) are more likely to exert mild and multi-target action with few side-effects [1]. Hence, TCMs may stand a chance as an alternative for the treatment of opioid addiction, considering that present medicine for opioid addiction cannot reverse the impaired neuronal system in addicts and possess adverse side-effects and abuse potential. TCMs and herbal medicines have been afforded increasing attention in addiction treatment [2-4]. AJN has been proved to be effective on protracted withdrawal symptoms in the clinic, although its mechanism of action remain to be fully elucidated [5, 6]. When evaluated from the angle of treatment outcome, TCMs can look even more favorable.

All the authors have checked the manuscript carefully to improve the language in this manuscript. The manuscript has also been re-edited by the language service WebShop.

The names in table 1 were Latin scientific names, We have checked and corrected them. As for the justification for TCM, we have make corrections in the context as follow:

Revised manuscript in Introduction section:

Traditional Chinese medicines (TCMs) have long been used to treat addictions, and many medicinal herbs and prescriptions were proved to have therapeutic effects (Mattioli et al., 2012; Shen et al., 2013; Shi et al., 2006).

References:


Additional revisions request from editor:
- Thank you for clarifying that Taier company played no role in your study beyond providing the herbal formula used. However, we would ask you to also declare this in your Competing Interests section.

Revised manuscript in Competing interests section:
The Taier company, manufacturer of AJN, played no role in this study beyond providing the herbal formula used.