Author’s response to reviews

Title: Anti-hyperalgesic properties of a flavanone derivative Poncirin in acute and chronic inflammatory pain models in mice

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POINT TO POINT COMMENTS AND RESPONSE

GENERAL COMMENTS: 1. The Authors have provided a thorough in vivo examinations of four types of inflammation mice pain model. What are the differences between these four inflammation models and/or how this strengthens Author's conclusion should be clearly stated (e.g. mimics the acute or chronic state of inflammatory pain). For example, Carrageenan induced two phases of edema with the first phase been the low-intensity edema. Ref: Mouse paw edema. A new model for inflammation? (PMID: 3690058).

This reviewer is asking Authors to clearly state the key differences between four types of inflammation mice pain model and based upon Authors' results to suggest the possible underlying mechanism/s of poncirin.

Response:
The reviewer is highly appreciated at this moments and in order to address the reviewer concern the difference between these four assay and poncirin potential anti-nociceptive mechanism have been described below. The formalin test is a valid model used in pain and analgesia research and has been reported to produce distinct biphasic nociceptive responses. The first phase (0 to 10 min) is believed to be caused by non-inflammatory pain resulting from direct stimulation of nociceptors. The second phase (10 to 30 min) is thought to be an inflammation-induced pain that is associated with inflammatory cytokines production. In general, centrally acting drugs inhibit both phases, whereas peripherally acting drugs inhibit only the second phase. The acetic acid-induced writhing test is a widely used classic non-selective animal model of pain. Acetic acid can produce peritoneal inflammation. The injection of acetic acid directly activates the visceral and somatic nociceptors that innervate the peritoneum and then induces inflammation not only in subdiaphragmatic visceral organs but also in the subcutaneous muscle walls. Furthermore, it is suggested that the nociceptive activity of acetic acid in the writhing model may be due to the release of inflammatory cytokines such TNF-α, IL-1β and IL-6, from the peritoneal macrophages. Similarly, these four pain models are also associated with the oxidative stress.

1. Zhi-YuYin et al., Antinociceptive effects of dehydrocorydaline in mouse models of inflammatory pain involve the opioid receptor and inflammatory cytokines. Scientific Report|6:27129|DOI: 10.1038/srep27129.


3. Ashok Kumar Gupta et al., Analgesic and Anti-Inflammatory Properties of Gelsolin in Acetic Acid Induced Writhing, Tail Immersion and Carrageenan Induced Paw Edema in Mice.


The carrageenan-induced models served as an acute inflammatory pain model and have been reported to be associated with the release of pro-inflammatory mediators and produ, which sensitize the nociceptors. Additionally, the CFA-induced model served as chronic inflammatory pain model and have been implicated with the release of pro-inflammatory cytokines. Taking together the poncirin anti-nociceptive activity in all these four animals is suggested due to the inhibition of the pro-inflammatory mediators, which serves to stimulate the nociceptor and initiate inflammatory process and pain.


Taking together, the poncirin might exhibit anti-nociceptive activity by modulating the inflammatory mediators and oxidative stress as mention in the conclusion section.

2. Method section, animals: What strain of mice? Also, typical body weight for mice at the age of 3-4 weeks should be ~20-25g. Please double check. If available xy fig for the animal body weights should be included as part of the result.

Authors did not provide the strain of mice. If the source is unknown, this need to be specifically stated.

Response:

The BALB/c mice were used in the current study. The strain of the mice was added in the revised manuscript as per recommendation of the reviewer.

3. Major concern: The behavioural readings (e.g. mechanical hyperalgesia and allodynia baselines) are much higher compared to other published data using mice. For example, Authors cited Cho H et al 2012 (ref #22), their von Frey and Randall Selitto baseline readings (Supplementary Fig6) are similar to this manuscript (~300g cf ~400g). However, Cho H et al use SD rats (Sfig6) not mice. Based on my knowledge and experience the behavioural baselines between rats and mice are distinctly different.

Thanks for providing six references. This reviewer, however, would like to point out that for references 1-5 they all use male SD rats not mice and so in fact supported this reviewer's original comment. The Authors should carefully select the supporting references for their rebuttal. For further reference, this reviewer would like to recommend Authors to provide a summary or in dot-points that support their claim. For examples:
1. Diana K. Schoeniger-Skinner et al., Interleukin-6 mediates low-threshold mechanical allodynia induced by intrathecal HIV-1 envelope glycoprotein gp120. Brain, Behavior, and Immunity 21 (2007) 660-667

* Adult male SD rats (300-450g).
* Using manual von Frey
* Their von Frey baseline readings before gp120-induced mechanic allodynia are ~10g.


* Adult male SD rat (200-350g)
* Manual von Frey
* Formalin-induced inflammatory pain
* Fig 1: pre-drug (formalin treated) baseline ~5g. Morphine treatment reverted von Frey reading back to ~13 g.


* Male SD rats (120-150g)
* Manual von Frey
* Pre-SNL surgery vF baseline ~15g (Fig 1A).
* Their vF value of 5.2 = 15.8g (under their animals and surgery section).

Reference #6 provided by the Authors is a paper that supported Authors' behavioural data.

* Male ICR mice (30-35g)
* Using manual von Frey and Randall Selitto with baseline readings of ~8g and ~400g respectively.
After google scholar search using e.g. ICR mice, Randall Selitto as keywords, I can only found the following paper that might support Authors' claim.

Effects of Astaxanthin from Litopenaeus Vannamei on Carrageenan-Induced Edema and Pain Behavior in Mice. DOI: 10.3390/molecules21030382

* Male ICR mice, eight weeks old, weighting 30-35 g,
* Baseline Randall Selitto value of ~350g (Figure 3). However, according to section "4.6. Behavioural Assessment of Mechanical Nociceptive Threshold Test", "The maximum force applied was limited to 200 g to avoid any tissue damage".

REQUESTED REVISIONS:

The behavioural data (especially, Randall Selitto and hot-plate data) are very different to the majority of published data. I still have my reservation on how a mice hindpaw can withhold >400g of force and last ~27 sec on a 55oC hot-plate. Authors should carefully select their supporting reference for their rebuttal.

Response:

The reviewer is highly appreciated at this moment. The hot plate temperature was maintained at 50±0.5 and the response was noted. The temperature was corrected in the revised manuscript. For the assessment of the mechanical hyperalgesia the sensitive Randall Selitto was used and it was tried level best to avoid any damage or harm to the paw tissue. In the present study, electronic Randall Selitto (the detail can be found on the web site i.e. http://www.iitcinc.com/Product%20pages/Analgesia/RandallSelitto.html) was utilized and it was very sensitive in nature, and the application of little force was associated with marked change in the threshold. The author tried their level best to avoid any damage to tissue or mice and followed the institutional ethical committee guidelines.

The purpose of providing the references was that there is great diversity among baseline reading threshold in rats as well as mice regarding the mechanical hyperalgesia and alldynia. The baseline threshold reported for rats are very high in some papers such as


Still, several studies suggest that the mechanical hyperalgesia and alldynia baseline threshold is reported very high.

1. Hiroki Ota et al., TRPV1 and TRPV4 Play Pivotal Roles in Delayed Onset Muscle Soreness. PLOS ONE | 2 June 2013 | Volume 8 | Issue 6 | e65751.
