Author's response to reviews

Title: Neuroprotective Efficacy and Therapeutic Window of Curcuma oil: In Rat Embolic Stroke Model

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Author's response to reviews: see over
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To,

Iratxe Puebla
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Dear Dr Puebla,

We are submitting our manuscript which has been revised keeping in mind the comments of your learned reviewers. We are giving here a point-by-point account of the changes made in the manuscript as per the comments of reviewers.

A colleague who has had English as a first language throughout his educational career has helped us to edit the paper. We were somewhat confused as three of the referees wanted the neurological data to be analyzed in three different ways, e.g.

1. Dr Eduardo Candelario-Jalil wanted the data to “be analyzed using a non-parametric statistical test, and presented as median/range”.
2. Dr Ashraf B Abdel-Naim was of the opinion that the data had better been analyzed “using a paired-wise test instead of the independent one way ANOVA”.


3. Dr Shyam S Sharma wanted the data “should be represented as median + 95% confidence limit and should be analyzed using Kruskal Wallis One way ANOVA on Ranks”.

We have followed the suggestion of Dr Eduardo Candelario-Jalil. We are willing to present the same data in three ways as per suggestions of the reviewers but it is normally not done.

We have tried to do our best to meet the major comments of the referees.

We were very disappointed by the comments made by Dr Shyam S Sharma, viz. “Even there are reports showing the neuroprotective potential of C. oil in cerebral ischemia. What this study adds to the existing knowledge of literature?” It would have been kind and helpful of him to have mentioned the relevant literature reference of his comments. We would be very grateful for the information. To the best of our knowledge, the first detailed report on the neuroprotective role of C. oil in stroke has been “Curcuma oil modulates the nitric oxide system response to cerebral ischemia/reperfusion injury” by Preeti Dohare et al. Nitric oxide. May 2, 2008, published online”. We are sorry; we are unable to accept the accusation.

With kind wishes,

Madhur Ray
Replies to the comments made by the Reviewers:-

Reviewer: Dr Eduardo Candelario-Jalil

1. “It would have been -----each brain region”.
Answer: We have not attempted to evaluate the region specific infarct volume. Total infarct volume of ipsilateral area is given in Fig.3 & 4.

2. “Why authors did not --------in both sides.’
Answer: The contra-lateral side of rat brain also shows some ischemia- induced changes, so this cannot serve as a control. In the present study, we wanted to compare the changes with the sham operated animal and not between the ipsilateral and contralateral sides of the same rat.

3. Fig. 3. “---- normalize------- neurons in ischemic tissue?’
Answer: We set the flowcytometer to acquire 10,000 events as that provides a 1% probability of error. We had not set it for the number of viable cells as that would have complicated the other estimations.

4. “How was the dose---------selected?”
Answer: The dose titration was done earlier and has been published in our earlier report. In the present paper, we have selected one dose and different time points after ischemia.

5. “What is ---------used in this study?’
Answer: The desired information has been incorporated. Please see page no 7 line 7-8.

6. “Was the --------- corrected for edema?’
Answer: This has now been done. Please see page 10 line 22 to page 11 lines 1 to 4

Fig.3&4.
7. “Neurological scores ----------- presented as median/range”.
   Answer: The data have been re-analyzed. Please see Fig 3(d) and 4(d)

8. “A major -------- infiltration of polymorphonuclear leukocytes.
   Answer: This problem had been taken care of and we have now described the detailed
   methodology for myeloperoxidase estimation. Please see page 12 lines 1 to 12

9. “Dihydrorhodamine -------------- also oxidized by H₂O₂”.
   Answer: The suggestion was accepted thankfully and the necessary corrections have been carried
   out. Please see page 14 line 18-22

10. “Western ---------- to actin or tubulin”.
    Answer: The suggestion was accepted gratefully and the necessary controls have been included.
    Please see Fig 7 (b), 8(b), 9(b) and 10 (a and b)

11. “How do -------- is not practical in this clinical condition”.
    Answer: The oral route has been used in certain cases (Joshi et al 2003 ref no 54). It may also be
    possible to introduce a stomach tube for administering the therapeutic agent. Other possible
    routes could be to introduce the agent high up in the rectum or administer the drug as a nasal
    spray. We have-not really addressed these problems in our work leaving it to other more
    specialized groups.

12. “There is a large -------- revise the manuscript.”
    Answer: As already mentioned above, the manuscript has been extensively revised. We hope the
    result will be found adequate.

13. “A list of abbreviations should be included”.
    Answer: A list has been included.
14. “Discussion should be shortened.”

Answer: The discussion has been shortened.

Reviewer: Dr Yasuhisa Furuichi

Reviewer's report:

Major Comments to the Author:

(1) The authors -----------clot-lysis action or not in this experimental condition.

Answer: The statistical analysis of the improvement seen in the lesion volume by MR imaging has been given. Please see Fig 2 (d). The clot lysing effect of C.oil has already been reported in our US patent .Please see Ray, M., Pal, R., Singh, S., Khanna, N. M., 2006. Herbal medicaments for the treatment of neurocerebrovascular disorders.
(http://www.freepatentsonline.com/6991814.html), United States (No: 6991814).

(2) It is unclear that -----well as 24 hours after ischemia

Answer: Please see page 25 lines 20-22.

(3) If the authors use the -------------- ischemia in this model.

Answer: The data has been provided. Please see Fig 3.

Minor comments:

(1) How did the authors ------------- this study?

Answer: The dose used in the present study has been selected on the basis of our previous report, viz., “Preeti Dohare, Saurabh Varma and Madhur Ray. Curcuma oil modulates the nitric oxide system response to cerebral ischemia/reperfusion injury. Nitric oxide. May 2, 2008, published online”.
(2) (P22, L23) Characters are blue.

Answer: It has been corrected.

(3) (Figure 3) In Y axis, DAF2-DA is wrong. “DAF-2DA” will be correct.

Answer: The necessary correction has been done.

(4) (P36, L3) In Figure legends, DHDCF-DA ----------” will be correct.

Answer: It has been corrected.

(5) (P38, L15) In Figure legends, Bax is wrong. “Bcl-2” will be correct.

Answer: It has been corrected.

**Reviewer: Dr Ashraf B Abdel-Naim**

Minor essential revisions

1. The authors conducted a large number of ----------------subject to erratic calculations.

Answer: Please see page 6, lines 1 to 14 and Fig 1

2. Why the control -------------------particular?

Answer: Please see page 7, lines 8 to 12.

3. On what basis such ------------------an optimal dose?

Answer: The dose was selected on the basis of our previous report “Preeti Dohare, et al. Nitric oxide. May 2, 2008, published online”.

4. Why the authors ------------------in brain lesions.

Answer: In the middle cerebral artery occlusion model where the occlusion is effected by filament, reflow time is certain i.e. when the occluder is removed. We have used the embolic
stroke model in the present study and in this model reperfusion is spontaneous. It has been suggested that embolic stroke model, encompassing a more ‘physiological’ spontaneous reperfusion process, more akin to the clinical pathology, would be more predictive with a low mortality rate and excellent reproducibility (Kudo, et al.1982 Stroke; 13:505-508.)

5. Figure 8 has dulled data armamentarium.
Answer: C.oil appears to exert its neuroprotective effects by acting at multiple targets in the signaling pathways that are activated in ischemia. We have tried to sum up the results for easier understanding by the readers.

6. Figure legends are details are available in methodology.
Answer: We have shortened the legends.

7. Authors emphasize of the oil (500 mg/kg).
Answer: There are possibly two ways of designing a study. One can use different doses and a particular point in time. That would be a way of finding an effective dose at that point of time. We have tried to find the longest time (or a fairly long time after the ischemic insult) when a reasonably small dose of the agent would give us a desired effect which can be measured fairly accurately.

8. Authors investigated the 1 h-point, even as a pilot trial?
Answer: We were unable to evaluate the neurological scores at 1 hr of reflow as the animals were still under the influence of the anesthesia (choral hydrate). Neurological responses under such conditions would not provide meaningful data.

9. Authors reported that have been more appropriate.
Answer: The sample of curcuma oil that we have used does not contain any curcumin. We have mentioned the chemical entities that are contained in C. oil. We have also mentioned pharmaco-kinetic data for the major components of C. oil. Please see page 6, lines 10-16.

10. As long as animals --------------independent one way ANOVA.

Answer: Neurological scores have been presented as median/range. Please see Fig 3(d) and 4 (d); please also refer to our reasons for doing so in our answers to points raised by Referee No 1(Dr E Candelario-Jalil).

11. Authors ----------------is to be addressed.

Answer: We could see neuroprotection till 5hrs post ischemia. A dose of 250mg/Kg body weight was effective when given before MCAo (for reference please sees the manuscript Dohare et al, 2008)

12. Text ----------------vocabulary.

Answer: The manuscript has been extensively proof-read.

13. What is the exact role of ----------------would be beneficial.

Answer: This aspect has now been covered in the Discussion Please see page 26 line 12-16.

Reviewer: Shyam S Sharma

Reviewer's report:

1. There are reports showing the neuroprotective potential of C. oil in cerebral ischemia. What this study adds to the existing knowledge of literature?

Answer: We are unable to find any such report in spite of diligent attempts made by us. Further, our patent (US and International) filed in the year 2002 (sealed 2006) is actually based on this
particular activity of Curcuma oil. We would be grateful if Dr S S Sharma can provide further references he may have come across.

2. Why late -------------------------using MRI.

Answer: The work was carried out in collaboration with an Institute. The work entailed long hours and we had no access to the particular instruments/equipment at these hours. We regret our inability to do the additional investigations.

We were unable to do this work.

3. Physiological ------------------ be provided with and without C. oil.

Answer: We have reported on the effects of C oil on blood pressure, heart rate in anesthetized animals. In these animals the body temperature was controlled artificially. We did not do these measurements on ischemic animals: We are sorry we do not have facilities for measuring blood gas status available with us.

4. Neurological deficit ----------------ANOV on Ranks.

Answer: Neurological scores have been presented as median/range as suggested by Dr E Candelario-Jalil(Reviewer No 1). Please see Fig 3(d) and 4 (d).

5. Brain area ------------------part in the photographs.

Answer: The points raised have been mentioned in the text.

6. Immunoblotting of ---------------------shown in the respective figure.

Answer: The necessary data have been provided. Please see Fig 7 (b), 8(b), 9(b) and 10 (a and b)

7. What the possible reason ----------C. oil treatment?

Answer: The section ‘Discussion’ covers these points. Please see page 29 line 4-17.
8. They have shown -----------------should be studied.

Answer: We have reported elsewhere on the dose-response profile of C oil. The present report is concerned with the time-lag (width of the time after ischemic insult) within which the drug has to be administered for a measurable beneficial effect.

9. Results are not --------------------------of some other study.

Answer: We have re-written the Discussion.