Author's response to reviews

Title: Brahmarasayana protects against Ethyl methanesulfonate or Methyl methanesulfonate induced chromosomal aberrations in mouse bone marrow cells

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Version: 2 Date: 19 April 2012

Author's response to reviews: see over
To
The Editor
BMC Complementary and Alternative Medicine

Dear Sir

Subject: Resubmission of Revised Manuscript
Reference: Manuscript ID 7789898066564739

With reference to the above, we are submitting the revised manuscript entitled ‘Brahmarasayana protects against Ethyl methanesulfonate or Methyl methanesulfonate induced chromosomal aberrations in mouse bone marrow cells’ for publication in your esteemed Journal. The authors are indebted to the reviewers for their valuable suggestions to improve the quality of our research paper. The comments of the reviewers are addressed and listed as point-by-point response below. We hope that you will find this revised manuscript sufficiently interesting and acceptable for publishing in your esteemed journal.

Kindly let us know if you have further queries

Thank you
Yours sincerely
Dr. K. Satyamoorthy
Answers to the referee’s comments: Dr. George Asare

1. Page 4. These abbreviations must first be defined: MAT-LyLu, VEGF, MMP-9 and MMP-2.
   Ans: Page 4 : Line 2 - 4: The sentence is modified as “…palpable tumours in adult malignant rat prostrate tumor cell (MAT-LyLu) inoculated Copenhagen rats [7] and repressing the production of pro-angiogenic factors like vascular endothelial growth factor (VEGF), matrix metalloproteinase 9 (MMP-9) and matrix metalloproteinase 2 (MMP-2)” as per referee’s suggestion.

2. Page 3. Brahmarasayana (BR), in abstract Brahma rasayana (BR) and Brahma rasayana (BR) or Rasayana (page 4 last line)
   Ans: Page 3: Brahmarasayana (BR), Brahma rasayana (BR) in abstract and Brahma rasayana(BR) or Rasayana (page 4 last line) is modified as ‘Brahmarasayana’ as per referee’s suggestion.

3. On page 5 the Rasayana group was fed with Rasayana 1 and Rasayana 2. However it is stated on page 4 ... Brahmarasayana was administered in the form of food pellets or by gavage. Where they given Rarasayana 1 or 2.....or both ?
   Ans: Page 5: Preparation and administration of rasayana: The Para is modified as “Brahmarasayana was administered in two different doses. The dose, based on body surface area of mice, was prepared in the form of food pellets, wherein 5 g of BR was mixed with 5 g of animal feed (henceforth known as Rasayana 1). This mixture was then pressed into pellets which, upon drying for a few hours, became hard and was ideal for chewing. The second dose, based on a body weight of 250 mg/kg body weight/day, was prepared by mixing approximately 8 mg of BR with sterile water (0.5 mL) and administering by gavage (henceforth known as Rasayana 2). Two different groups of animals were fed daily for 60 days with either Rasayana 1 or Rasayana 2”.

4. Treatment schedule on page 5 needs clarity. Clastogen treatment: Animals were divided into two groups (n = 9) based on their treatment with either EMS (240 mg/kg body weight) or MMS (125 mg/kg body weight). n=9 is that the total of 9 each? For the control group you stated that it was for 60 days. The number of days for the Clastogen treatment group should be stated.
   Ans: Page 5 - 6: Treatment Schedule: Clastogen treatment: The sentence is changed to “Animals were divided into two groups and maintained for 60 days with regular food. On day 61, one of the groups (n = 9) were injected with EMS (240 mg/kg body weight) whilst the other group (n = 9) with MMS (125 mg/kg body weight).”

5. Page 5. Rasayana and EMS- or MMS-combined treatment: Is this a new treatment group; I believes so. What number constituted this group?
Ans: Page 5- 6: **Treatment Schedule: Rasayana and EMS- or MMS-Combined treatment:**
To answer the reviewer’s query, it was a new group of animals (n = 9) and the paragraph is modified to reflect accordingly, as follows: “Two groups of animals (n = 9 for each group) were fed with Rasayana 1 (Group 1) and Rasayana 2 (Group 2) for 60 days. After 60 days, one group of animals belonging to Rasayana 1 and Rasayana 2 were injected with EMS and the other group with MMS at the same dose as injected for the clastogen treatment group alone”.

6. Page 5. Under Treatment schedule...line 2...were fed with ....should change to.....were fed....
Ans: Page 5 - 6: **Treatment Schedule: Rasayana:** “were fed with” is changed to “were fed” as per referee’s suggestion.

7. Page 5. the animals were injected...........were they injected i.v, i.m. or i.p? and at what site? The same applies to 0.05% colchicine (0.5 mL) injection.
Ans: Page 6: **Mitotic preparation and chromosome analysis:** The sentences were modified as “Animals were sacrificed at 24h, 48h and 72h after the treatment of clastogen or 0.09% NaCl by i.p. injection. Colchicine (0.05% : 0.5 mL), a spindle fibre inhibitor that arrests cells at metaphase was also administered by i.p. injection 90 minutes prior to sacrifice of these animals.” as per referee’s suggestion.

8. Page 5. Three recovery times (RTs) of 24h, 48h and 72h were employed for all treatment procedures. Which treatment procedures are you referring to? Is it the treatment schedule? Furthermore define recovery time...........recovery time from what. Bear in mind that the statement that follows deals with sacrifice. It is very confusing whether they were first injected with 0.05% colchicine (0.5 mL) and allowed to recover.
Ans: Page 6: **Mitotic preparation and chromosome analysis:** The sentence Three recovery times (RTs) of 24h.....procedures is changed to “Animals were sacrificed at 24h, 48h and 72h after the treatment of clastogen or 0.09% NaCl by i.p. injection. Colchicine (0.05% : 0.5 mL), a spindle fibre inhibitor that arrests cells at metaphase was also administered by i.p. injection 90 minutes prior to sacrifice of these animals” as per referee’s suggestion.

9. Page 5. Mitotic preparation and chromosome analysis. Was this done on a different set of animals? if so, how many?
Ans: Page 6: Mitotic preparation and chromosome analysis were done on same set of animals.

10. Page 5. The reason for injecting with the choice of drug colchicine, has not been stated.
Ans: Page 6: **Mitotic preparation and chromosome analysis:** The reason for using colchicine is included in the sentence as above (Point No. 7 and 8).

11. Page 6. “The bone marrow was then flushed with 0.56% KCl (5 mL) with the help of a gavage attached to a syringe (5 mL).” Is it a gavage needle or gavage. The word gavage refers to forced feeding.
Ans: Page 6: Our apologies for the error. **Mitotic preparation and chromosome analysis:**
The sentence is modified as “**In brief, the femur bones were dissected and flushed with 0.56% KCl (5 mL) with the help of a 26 gauge needle attached to a syringe (5 mL).**”

Ans: Page 6: **Mitotic preparation and chromosome analysis:** The reference is included for documented standard protocol for mitotic preparation and chromosome analysis as -;
“….by routine standard air dry technique [11]” as per referee’s suggestion.

13. Page 6. Liver homogenate is prepared....However, there is no due reference to liver harvesting at sacrifice and how the liver was stored.
Ans: Page 7: **Base excision repair** : The reference to animals was included as per reviewers suggestion as follows: “Approximately 1g of liver from control, Rasayana 1 and Rasayana 2 groups of mice was homogenized with PBS (pH = 7.4, 10 mL) immediately after the sacrifice to obtain a 10% homogenate”..

14. Page 7. GSH is an antioxidant but not an enzyme. The title should therefore read........, Antioxidant analyses and not enzyme analyses.
Ans: Page 7: Our apologies for the error. The title “enzyme analyses” is changed to “Antioxidant analyses” as per referee’s recommendation

15. Page 7. Sperm count is mentioned for the first time with due reference in the methodology of organ or tissue sampling or sperm collection, at the time of sacrifice.
Ans: Page 8: We thank the referee for pointing out this omission. Methodology for sperm count and sperm abnormalities under the title Reproductive toxicity is now included.

16. Page 8. “However in case of MMS treatment, combined treatment of rasayana group I and MMS showed.....””. Group 1 is mentioned for the first time without such designation in the Methods section.
Ans: Page 8 - 9: **Results:** “However….. Group1” is modified and Group 1 is referred to as “Rasayana 1”

17. Page 9. “...On the other hand, there was no significant variation in the GSH activity in all the treatment.” Since GSH is not an enzyme you it is better to mention GSH level or concentration, rather than activity.
Ans: Page 10: **Results:** “On the other hand..... treatment” is changed to “On the other hand, there was no significant variation in the GSH level in all the treatment groups” according to referee’s recommendation.

18. Page 10. and page 11, line 7 (plus others in the discussion)...on par with our earlier results........should change to .....on a par.....
Ans: Page 10 - 14: **Discussion:** “On par with our earlier results” is changed as per the referee’s comment.
19. Page 16. Legends for figures do not describe the events. They are mere titles. Better descriptions of the figures are needed.
Ans. Page 19: **Legends**: Descriptions of the figures are given as per reviewer’s suggestion.

20. Page 17. Column must be created for the p-values in Tables 1, 2 and 3.
Ans: Page 20: **Tables**: Column for p-values (Significance was indicated with an asterisk) in Tables 1, 2, and 3 are inserted as per referee’s comments.

21 Figures 18 A & B. The figures are very poor. The key to the graphs indicate 6 independent variables. However, only 4 are shown the figures. Graphs for control and Rasayana 1 are missing.
Ans: **Figures 1A and B**: control and rasayana 1 are missing? – The level of chromosomal aberration in control, rasayana1 and rasayana 2 are almost same in all the time points tested indicating no chromosomal aberrations induced by rasayana. In the Figure, as the values are almost same, the lines are merged. The Figures are modified to discriminate control, rasayana 1 and rasayana 2 as per the referee’s suggestion.
Answers to the referees comments: Dr. Papiya Bigoniya

Major Compulsory Revisions

1. Justification to the selected experimental protocols in concern to the targeted aim and objective of the study in not well defined in the Introduction as well as in the Discussion section.

Ans: Page 4: **Background**: A) Justification to the selected experimental protocols in concern to the targeted aim and objective of the study was discussed in Introduction as “It is well known that the genome of all the organisms including human is susceptible to damages caused by vast array of both endogenous and exogenous DNA damaging agents. Repair of such DNA damages play an important role in cellular functions. Defects in DNA repair or inefficiency of DNA repair machinery results in accumulation of DNA damages in the genome which in turn are responsible for various disorders including cancer and ageing. Although various beneficial effects of Brahmarasayana was investigated, there are no reports to date of BR on potentiation or protection of DNA repair. Hence, the present study was undertaken to evaluate the effect of BR on chromosomal aberrations induced by ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS), two well-known mutagenic and clastogenic agents in *in vivo* mouse test system. Furthermore, the effect of BR on constitutive base excision repair has also been undertaken to understand its role as a repair enhancer”.

B) Page 10: **Discussion**: Justification on aim and objectives was discussed as per reviewer’s suggestion. Accordingly it is put as follows. “It is beyond doubt that DNA is the primary target of several chemicals and physical agents which cause the genetic alterations viz. point mutations, chromosomal aberrations, SCEs and others. These induced alterations in the DNA, if accumulated leads to various health hazards including cancer. However, there are several mechanisms to repair the damaged DNA in the living organisms, which are responsible to maintain the stability of genetic material. Defective or inefficient repair machinery may not combat efficiently the genotoxic effects induced by the agents. Furthermore, the efficiency of DNA repair machinery may also be comparable to rate of repair / removal of DNA damages. Therefore, we hypothesize that the DNA repair efficiency may be modulated by rasayanas. Hence, in the present investigation, an attempt was made to understand the effect of Brahmarasayana, a well-known Medhya rasayana on EMS- and MMS- induced chromosomal aberrations / repair of chromosomal aberrations in an *in vivo* mice system”.


2. Procurement of study drug material (Brahmarasayana) should be authenticated with manufacturing date and date of expiry. Use of study material from five different batches must be justified with need, length of study, collection and processing months as well as consistency of phytoconstituents. Presentation of TLC, HPLC or HPTLC fingerprinting data may be used for standardization and authentication of the product.

Ans: Page No 5: **Chemicals**: Justification was mentioned in the script as per the reviewer’s recommendations. To answer the reviewer’s query Brahmarasayana is commercial product. The date of manufacture and expiry has been noted. The HPTLC profile of batch specific products were obtained and placed on record for the authentication and comparison purpose. “Brahmarasayana a commercially available product prepared by Arya Vaidya Sala (Kotakkal, India) was purchased from local Ayurvedic vendors. The manufacture and expiry date of each batch (Batch Nos. 501003, 503232, 503539, 503772 and 504015) was noted. In addition, the HPTLC profile of batch specific product was obtained from the manufacturer and placed on record for authentication and comparison purposes”. These data are available upon request.

3. Specify what is rasayana 1 and 2.

Ans: Page No 5: **Preparation and administration of Brahmarasayana** - Rasayana 1 is the dose of Brahmarasayana calculated based on body surface area of mice and Rasayana 2 is the dose of Brahmarasayana calculated based on body weight. These were specified under the title “Preparation and administration of Brahmarasayana” as per referee’s recommendations.

4. What is the rationale behind selection of the dosing schedule? Give justification with scientific data support. Justify the safety profile of rasayana 1 and 2 under study for dosing upto 60 days (Subacute dosing safety profile).

Ans: Page 10: **Discussion**: Justification in relation to rasayana 1 and 2 under study for dosing up to 60 days was discussed as per referee’s suggestion.

5. In some places of the Discussion section, the results has repeated.

Ans: Page 10 – 11: **Discussion**: Discussed as per the referee’s recommendations.

6. Discussion should only include major findings with proper cited justification

Ans: Page 10 -14: **Discussion**: Major findings were discussed as per reviewer’s recommendations.

7. The therapeutic implication of the found data in concern to greater interest of patients and scientific community should be discussed. The study limitations should be defined clearly in the conclusion section.

Ans: Page 14: **Conclusions**: A) The therapeutic implication of the observed data in concern to greater interest of patients and scientific community is discussed as per reviewer’s suggestion. Accordingly the following sentence is included --“The study provides *in vivo* evidence in mice for the anticlastogenic activity of BR and hence a potential for
Further evaluation in humans on the protective effects of such formulations in cancer treatments where patients receive high doses of radiation and chemotherapy and in anti-aging research.

B) Page 15: Conclusions: The limitations of the study are added as per the Recommendations of reviewer as follows: "The study is limited at present to evidence generated from studies on mice and further exploration is therefore required to establish if similar effects are seen in humans."

8. No phytochemical (qualitative or quantitative) estimations has been performed to support phyto-pharmacological mechanistic correlation. In conclusion section presence of some phytochemicals has been claimed citing the compendium only for the plant constituents of rasayana. This needs citation of scientifically published data on presence of these constituents in the study material itself (Brahmirasayana).

   Ans: The study was not focused on the phytochemical estimations or particular mechanistic correlations but rather on the activity of BR which is prepared following a specific recipe. Follow-up studies on phytochemical and mechanistic aspects are however very important and forms part of the ongoing research into Brahmarasayana. In conclusion section presence of some phytochemicals has been claimed citing the compendium only for the plant constituents of rasayana. This needs citation of scientifically published data on presence of these constituents in the study material itself (Brahmirasayana). – The citations “[39 -43]” are included as per referee’s suggestion.

9. The free radical predominately produced during cellular damage is hydroxyl radical, which needs to assessed for proving rationale of chromosomal aberration protectors.

   Ans: Page 11: Discussion: The chromosomal aberrations were induced by different agents and different mechanisms. Hence, it was discussed as follows: “There are different mechanisms which lead to chromosomal aberrations such as mis-repairing of double strand breaks [23], stickiness and direct breakage of phosphor di-ester bonds of DNA by alkylating agents [24] and others which rely on DNA damaging agents: and free radical generation induced DNA damage is one such mechanisms. The future work needs to be focused on unraveling the mechanistic pattern of cellular damage with respect to free radicals and alkylating agent- induced chromosomal damage.”

10. Positive control (standard drug treated) group has not been included in the study to compare the level of significance of data. Justify?

    Ans: Positive control group is included in the studies. Clastogen treatment group is the positive control (EMS or MMS only), where-as the animals, that received regular food for 60 days and 0.9% NaCl i. p. injection form negative control. These are mentioned in the script as per reviewer’s suggestion.

Minor Essential Revisions
1. Mention the status of Institutional Animal Ethical Committee approval for the study protocol. Housing conditions of the animals are to be defined in the Animal section of Methods heading.

   Ans: Page 4-5: **Methods: Animals**: The sentence “The study was approved by the Institutional animal ethics committee (Kasturba Medical College, Manipal) and all…” is inserted as per referee’s suggestion.

   Page 4: **Methods: Animals**: Housing conditions of the animals are now mentioned as “They were maintained in polypropylene shoebox cages with a grill top. Paddy husk was used as bedding material and the animals were fed with standard diet and water ad libitum. Three animals were housed per cage and utmost care was taken to maintain cage hygiene and also provide good ventilation and aeration in the room where the animals were housed”

2. Mitotic preparation and chromosome analysis study protocol has not been supported by reference citation?

   Ans: Page 5-6: The reference citation is included as per referee’s recommendations.

3. The level of p-value significance should be marked in the respective columns of the tables.

   Ans: Tables: The level of p-value significance is included as per referee’s comments.

4. Toxicant and combination groups are absent in the figure 2.

   Ans: **Figure 2**: The constitutive base excision repair was analyzed only in control and rasayana treated animals to understand the role of rasayana on BER. It was not carried out in toxicant and combination groups and the data explains beneficial role of rasayana on constitutive BER. This is discussed in the Discussion section also as “We did not assess the BER capacity in EMS and MMS-challenged animals or animals treated with BR and EMS or MMS-combined treatments as they already possess inherently high levels of DNA damage and repair and it is difficult to discern differences in BR treated and clastogen only treated groups”.

**Other Changes made in the manuscript:**

1. Page 2: **Abstract: Methods**: ‘Brahma rasayana (BR)’ is changed to ‘Brahmarasayana’.

2. Page 2: **Abstract: Results**: ‘Rasayana’ is changed to ‘BR’

3. Page 4: **Methods: Treatment Schedule: Control**: The sentence “On day 61, these animals received 0.9% NaCl solution (0.5 mL) as intraperitoneal (i.p.) injection” is inserted.

4. Page 8: **Results**: Line 3: ‘Rasayana’ is changed to ‘BR’
5. Page 8: **Results**: Line 5: ‘(EMS; 240 mg / kg body weight)’ is modified as *(EMS; 240 mg / kg body weight; positive control).*

1. Page 8: **Results**: Line 10: ‘Rasayana’ is changed to ‘BR’.

2. Page 9: **Results**: Line 1: “positive control” is inserted

3. Page 9: **Results**: Line 5: ‘Rasayana’ is changed to ‘BR’.

4. Page 9: **Results**: Line 8: ‘Rasayana’ is changed to ‘BR’.

5. Page 9: **Results**: Line 14: ‘mitotic cells in rasayana group 1’ is changed to ‘mitotic cells in animals treated with Rasayana 1’.


8. Page 9: **Results**: Line 28: ‘SOD level in Rasayana group 1’ is changed to ‘SOD level in Rasayana 1’


11. Page 10: **Results**: Line 4: Rasayana group 1’ is changed to ‘Rasayana 1’

12. Page 10: **Results**: Line 6: Rasayana group 2’ is changed to ‘Rasayana 2’

13. Page 12: **Discussion**: Line 25: The sentence “This is understandable as cells with a large number of breaks stop cell cycle” is inserted.

14. Page 12: **Discussion**: Line 26: Rasayana group 1’ is changed to ‘Rasayana 1’

15. Page 13: **Discussion**: Line 1: The sentences “This particular finding validates the use of two dosing regimens. We find that Rasayana 1, in addition to being more protective, also induces greater mitotic cellularity. Utilizing human equivalent doses (as in Rasayana 1) of Ayurvedic formulations for animal studies present a more realistic outcome in terms of translation and by this notion BR elicits better protection, at least in the mice treated with Rasayana 1” are inserted.

16. Page 13: **Discussion**: Line 17: The sentence “These results are on par with the earlier results” is modified as “These results are in accordance with our earlier findings [9].”

17. Reference: (Reference No. 11, 17, 18, 23, 24, 39, 40,41,42 and 43 were included in the reference list. The Journal name is modified as per the Journal style. The citation numbers in the script are changed accordingly.

18. Legends: Table 1: ‘Rasayana and EMS or MMS treated’ is changed to ‘Brahmarasayana and EMS- or MMS-treated’. 
19. Legends: Table 2: ‘Rasayana’ is changed to ‘Brahmarasayana’.

20. Legends: Table 3: ‘Anti oxidant enzyme activities in rasayana and EMS or MMS treated animals’ is changed into ‘Anti-oxidant levels in Brahmarasayana and EMS- or MMS-treated animals’.