

March 17, 2015

Dear Prof. Damian Garcia-Olmo, Prof. Saleh A Naser, Prof. Stephen C Strom, and Prof. Andrzej S Tarnawski,

Please find enclosed the edited manuscript in Word format (file name: 16159-Basic Study.doc).

**Title:** Brewers' rice modulates oxidative stress in azoxymethane-mediated colon carcinogenesis in rats

**Authors:** Bee Ling Tan, Mohd Esa Norhaizan, Ky Huynh, Swee Keong Yeap, Hamzah Hazilawati, Karim Roselina

**Name of Journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO:** 16159

The manuscript has been improved according to the suggestions of reviewers and editor:

1. Format has been updated.
2. Revision has been made according to the suggestions of the reviewer no.:
  - (1) 3004091
  - (2) 189327
  - (3) 58696
  - (4) 2438232
3. References and typesetting were corrected.

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,



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## LIST OF CORRECTIONS

Reviewer No.: 3004091

Major comments

No	Comments from the reviewer	Correction made	Page no.
1.	<p>The effect of different dosages of brewer's rice (10%, 20% and 40%) is different for different parameters e.g. though 40% diet had maximum effect on GSK3beta and beta-catenin, 10% showed the maximum effect on NF-kB. No explanation has been provided by the authors for these.</p>	<p>As explained in the paragraph.</p> <p>"In the present study, treatments with brewers' rice resulted in increased <i>GSK3β</i> and decreased <i>β-catenin</i>, and the maximum effect was observed with 40% (w/w) brewers' rice. The effects observed in the treatment with 40% (w/w) brewers' rice could be explained by its higher concentrations of active compounds in brewers' rice, which may confer better functional properties in the regulation of Wnt/<i>β-catenin</i> signaling pathway".</p> <p>"Although the maximum effect was observed in 10% (w/w) brewers' rice, there was no significant difference between groups fed with 10% (w/w) brewers' rice and groups fed with diets containing 20% (w/w) brewers' rice or 40% (w/w) brewers' rice (<math>p &gt; 0.05</math>). The reason for the lack of any clear dose-dependence effects remains to be elucidated. One of the possible reasons may be due to the efficiency of brewers' rice involved in the inhibition of <i>NF-κB</i> transcriptional activity reached with 10% (w/w) brewers' rice".</p>	<p>Page 13 and page 14</p>
2.	<p>The statistical significance is difficult to understand as it does not describe clearly the</p>	<p>The interpretation of statistical significance was stated as a footnote in the figures and table.</p>	<p>-</p>

	comparison among the groups.	“Values with different superscript letters indicate significant differences between groups by Tukey’s test ( $P < 0.05$ )”.	
3.	The results of iNOS does not correlate with NO levels. The authors have mentioned in their discussion that iNOS is responsible for NO production, however, their results on this are contradictory. No clearcut explanation for this has been given in the discussion section.	<p>The explanation was provided in the ‘DISCUSSION’ section.</p> <p>“Our study shows contradictory results. It is possible that multiple cellular factors affect the sensitivity of NO, like specific NO metabolism pathways and interactions with other free radicals. The sensitivity of NO may also be associated with the expression of apoptosis-associated proteins, including Bcl-2, Bax, and Fas<sup>[30]</sup>”.</p> <p>References  30. <b>Xie K</b>, Huang S. Contribution of nitric oxide-mediated apoptosis to cancer metastasis inefficiency. <i>Free Radic Biol Med</i> 2003; <b>34</b>: 969-986 [PMID: 12684082 DOI: 10.1016/S0891-5849(02)01364-3]</p>	Page 14
4.	The reason behind increased NF-κB expression in 20% and 40% brewers’ rice diet has not been explained properly.	<p>The reason was provided in the ‘DISCUSSION’ section.</p> <p>“Although the maximum effect was observed in 10% (w/w) brewers’ rice, there was no significant difference between groups fed with 10% (w/w) brewers’ rice and groups fed with diets containing 20% (w/w) brewers’ rice or 40% (w/w) brewers’ rice (<math>P &gt; 0.05</math>). The reason for the lack of any clear dose-dependence effects remains to be elucidated. One of the possible reasons may be due to the efficiency of brewers’ rice involved in the inhibition of <i>NF-κB</i> transcriptional activity reached with 10% (w/w) brewers’ rice”.</p>	Page 14

### Minor comments

No	Comments from the reviewer	Correction made	Page no.
1.	The methods section does not clearly state the number of dosages of AOM given. It should be clearly stated.	The number of dosage of AOM given was stated in 'MATERIALS AND METHODS' and abstract under 'METHODS' section. "They were intraperitoneally administered 15 mg/kg body weight of azoxymethane (AOM) in saline once weekly over a two-week period".	Page 4 and page 7
2.	There is no major difference between the levels of SOD using 20% and 40% brewer's rice and therefore, the statement stating that highest dose of brewer's rice shows a value closest to the normal is erroneous.	The statement was removed from the paragraph.	-

Minor comments

No	Comments from the reviewer	Correction made	Page no.
1.	ABSTRACT: in the aim, the authors say "regulating the gene expression" (which one?).	The sentence was revised. "To investigate the mechanistic action of brewers' rice in regulating the <b>Wnt/NF-κB/Nrf2-signaling pathways</b> during colon carcinogenesis in male Sprague-Dawley rats".	Page 4
2.	Results: the sentence related to NF-κB mRNA level should be re-written. It is not clear.	The sentence related to NF-κB mRNA level was revised to " <b>NF-κB expression</b> ".	-
3.	RESULTS:1) NF-KB. Why there is none in normal tissue?	<p>"NF-κB plays a crucial role in carcinogenesis and inflammation (Maeda and Omata, 2008). In the present study, none of the <i>NF-κB</i> expression was observed in the normal group. These findings were consistent with a study reported by Kim <i>et al</i> (2009), who found that no inflammatory score was obtained in untreated group (normal group). These results also might be due to the <i>NF-κB</i> expression in the normal colon tissue was too low to be detected by quantitative real-time PCR analyses. This suggests that the colon tissues may be insensitive to the events that do not affect metabolically in normal colon mucosa. Alternatively, it is possible that the expression of NF-κB may occur transiently and for only a short duration, making the detection difficult".</p> <p>References  <b>Kim</b> M, Miyamoto S, Yasui Y, Oyama T, Murakami A, Tanaka T.</p>	-

	<p>Why the maximum effect was with 10 % brewers' rice? Values with different concentrations were the same.</p>	<p>Zerumbone, a tropical ginger sesquiterpene, inhibits colon and lung carcinogenesis in mice. <i>Int J Cancer</i> 2009; <b>124</b>: 264-271 [PMID: 19003968 DOI: 10.1002/ijc.23923]</p> <p><b>Maeda S</b>, Omata M. Inflammation and cancer: role of nuclear factor-kappaB activation. <i>Cancer Sci</i> 2008; <b>99</b>: 836-842 [PMID: 18294278 DOI: 10.1111/j.1349-7006.2008.00763.x]</p> <p>“Although the maximum effect was observed in 10% (w/w) brewers’ rice, there was no significant difference between groups fed with 10% (w/w) brewers’ rice and groups fed with diets containing 20% (w/w) brewers’ rice or 40% (w/w) brewers’ rice (<math>P &gt; 0.05</math>). The reason for the lack of any clear dose-dependence effects remains to be elucidated. One of the possible reasons may be due to the efficiency of brewers’ rice involved in the inhibition of <i>NF-κB</i> transcriptional activity reached with 10% (w/w) brewers’ rice”.</p>	<p>Page 14</p>
<p>4.</p>	<p>What explanation the authors have with the 20% concentration, mainly on Nrf2 mRNA.</p>	<p>“Our results showed that the group treated with 20% (w/w) brewers’ rice effectively activates the gene expression of <i>Nrf2</i> compared to 10% (w/w) brewers’ rice. The activation of <i>Nrf2</i> seen in the concentration of 20% (w/w) of brewers’ rice treatment could be explained by its higher concentrations of active compounds in brewers’ rice that may confer better functional properties in regulation of <i>Nrf2</i> signaling pathway. However, <i>Nrf2</i> mRNA level in the group treated</p>	<p>-</p>

		with 40% (w/w) brewers' rice was significantly lower compared to 20% (w/w) brewers' rice. These observed effects may be due to the efficiency of brewers' rice involved in the upregulation of <i>Nrf2</i> mRNA level reached with 20% (w/w) brewers' rice".	
5.	English style might be improved.	The manuscript has been sent to the American Journal Experts before submitted to World Journal of Gastroenterology.	-

Reviewer No.: 58696

Major comments

No	Comments from the reviewer	Correction made	Page no.
1.	Although the authors have proposed a study aim, there is no clearly outlined hypothesis.	The hypothesis is stated in the 'INTRODUCTION'. "We hypothesized that brewers' rice may provide chemopreventive or chemotherapeutic effects against colorectal cancer via regulation of multiple signaling pathways".	Page 6
2.	It is not delineated whether the authors and/or individuals interpreting the results were blinded to the five groups.	Yes, the authors were blinded when interpret the results among the five groups.	-
3.	Neither the sources of funding, nor potential conflicts of interest are disclosed.	The authors declare that they have no conflicts of interest, as stated in the title page.	Page 2
4.	The authors have not clearly stated whether there was an institutional review board (IRB) approval for this study.	This study was conducted following the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM) Serdang, Selangor (IACUC protocol number: UPM/FPSK/PADS/BR-UUH/00461). This statement has been stated in the 'diet and animals' under 'MATERIALS AND METHODS' section.	Page 7
5.	The authors do not specify the limitations of their study (such as being conducted in only male rats), or suggest areas of further study which are needed.	The limitations of the study and recommendation of future study were stated in the 'Conclusion' section. "This study has been limited to the use of brewers' rice in male Sprague-Dawley rats, and the duration of the treatment was only twenty weeks. Therefore, further	Page 16

		studies are warranted in long-term animal studies or human clinical trials to confirm these findings".	
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### Minor comments

No	Comments from the reviewer	Correction made	Page no.
1.	In the abstract section under 'Methods', the terms 'AOM' and '(w/w)' are used prior to being defined.	The terms 'AOM' and '(w/w)' were defined. "azoxymethane (AOM) and (weight (w)/weight (w))"	Page 4
2.	In fact, '(w/w)' is never clearly defined in the study.	The term '(w/w)' was defined in the 'MATERIALS AND METHODS' section under 'diet and animals'. "(weight (w)/weight (w))"	Page 7
3.	In the 'Conclusion' section the authors could clarify the role of 'Nrf2 and Wnt/NF-κB' in colon carcinogenesis.	The roles of 'Nrf2 and Wnt/NF-κB' were stated in the 'Conclusion' section. "Uncontrolled signaling through the wntless/Wnt pathway and overexpression of NF-κB have been reported to play crucial roles in the development of colorectal cancer. Nrf2 is responsible in the regulation of phase II detoxification and antioxidant enzymes".	Page 16
4.	On page 5 under 'Introduction', lines 2-3, the authors do not specify if these statistics refer to the United States or worldwide.	These statistics refer to the worldwide as stated in the 'INTRODUCTION'.	Page 6
5.	On page 5, line 16, consider changing 'remedies' to 'remedy'.	The grammatical error was corrected. The word 'remedies' has been changed to 'remedy'.	Page 6
6.	On page 5, line 27, consider striking "of brewers' rice due to their prominent antitumor activities".	This sentence has been removed from the paragraph.	-

7.	On page 5, line 28 consider changing 'mechanisms' to 'mechanism underlying these effects remain obscure.'	The grammatical error was corrected. The word 'mechanisms' has been changed to 'mechanism'.	Page 6
8.	On page 5, line 28 to page 6 line 1, consider striking "Therefore, in the present study, we determined..." to "The present study sets out to determine..."	The grammatical error was corrected. The sentence "Therefore, in the present study, we determined..." has been changed to "The present study sets out to determine..."	Page 6
9.	On pages 7-11, in the 'Materials and Methods' section consider summarizing the information further and placing detailed procedural information such as 'Chemicals and reagents, brewers' rice', 'diet and animals', 'PCR analysis', 'Colon tissue preparation' in an 'appendix section' rather than in the 'Methods' as they detract from overall fluency of the paper.	We followed the writing format of basic study provided by 'Baishideng®', for example 'animal model', 'tissue handling', and 'PCR analysis', in which they are placing in the 'MATERIALS AND METHODS' section.	-
10.	On pages 12-14, consider explaining specific values obtained and results from figures/tables in detail in the body of the publication.	The specific values obtained and results from figures or tables were included in the 'RESULTS' section as suggested.	Pages 11-12
11.	On pages 15-21, consider summarizing the discussion further as it is a lengthy discussion of the prior work on the subject area and touches on subject matter previously referred to in the publication.	The discussion has been shortened.	Pages 13-16

Reviewer No.: 2438232

Major comments

No	Comments from the reviewer	Correction made	Page no.
1.	The aim of the abstract part is not Accurate.	The aim in the abstract was revised. "To investigate the mechanistic action of brewers' rice in regulating the Wnt/NF- $\kappa$ B/Nrf2-signaling pathways during colon carcinogenesis in male Sprague-Dawley rats".	Page 4
2.	In the introduction, the author should describe why GSK3 $\beta$ , iNOS, Nrf2, and HO-1 genes was chosen in this paper. Some studies had introduced the relationship among these genes.	The reason why these genes were chosen was provided in the 'INTRODUCTION'. "The present study sets out to determine whether brewers' rice confers suppressive effects on the gene expression of $\beta$ -catenin and key inflammation markers, such as NF- $\kappa$ B and iNOS, which are particularly critical in the development of colon cancer. Glycogen synthase kinase 3 $\beta$ (GSK3 $\beta$ ), a destruction complex that modulates the degradation of $\beta$ -catenin, was also evaluated. Moreover, the potential roles of brewers' rice in the regulation of Nrf2-dependent transcriptional activity were assessed during AOM-induced colon tumorigenesis in male Sprague-Dawley rats. Nrf2 and heme oxygenase-1 (HO-1) were evaluated to determine the effect of brewers' rice in carcinogen metabolism against detoxification".	Page 6
3.	Western blot should be performed to confirm the results.	In the present study, the glycogen synthase kinase 3 $\beta$ (GSK3 $\beta$ ), $\beta$ -catenin, key inflammation markers, nuclear factor E2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) were evaluated by quantitative real-	-

		time PCR analyses, and perhaps this is the limitation of the study. Further studies are required to confirm these findings through western blotting analyses.	
4.	<p><math>\beta</math>-catenin may translocate from the cytoplasm to the nucleus, where it might serve as a transcriptional factor to stimulate tumour formation. Thus, only <math>\beta</math>-catenin expression was not enough. Immunofluorescence should be performed to study the location change of <math>\beta</math>-catenin.</p>	<p>The expression of <math>\beta</math>-catenin was evaluated in our previous study using immunohistochemical analysis. We demonstrated that the abundance of <math>\beta</math>-catenin was primarily present in the cytoplasm and a relatively high nuclear staining was observed in the group administered with AOM. However, administration of brewers' rice reduced the expression of <math>\beta</math>-catenin in both the cytoplasm and the nucleus (Tan et al., 2014).</p> <p>Reference  <b>Tan BL</b>, Norhaizan ME, Heshu SR, Hazilawati H, Roselina K. Brewers' rice induces apoptosis in azoxymethane-induced colon carcinogenesis in rats via suppression of cell proliferation and the Wnt signaling pathway. <i>BMC Complement Altern Med</i> 2014; <b>14</b>: 304 [PMID: 25129221 DOI: 10.1186/1472-6882-14-304]</p>	-