Author's response to reviews

Title: Histological analysis of low dose NMU effects in the rat mammary gland

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Author's response to reviews: see over
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John Kerr
Assistant Editor
Biomed Central

Dear Dr. Kerr,

Enclosed, please find the revised manuscript number 1190125175254383 previously titled, “N-nitrosomethylurea in the mammary gland: is there a non-carcinogenic dose?” by Tessa J. Murray, Angelo A. Ucci, Maricel V. Maffini, Carlos Sonnenschein, and Ana M. Soto. The revised title is, “Histological analysis of low dose NMU effects in the rat mammary gland”.

This manuscript reports our findings after conducting an NMU dose-response experiment in the susceptible Wistar-Furth strain where rats were exposed to 10, 20, 30 or 50mg NMU/kg body weight (BW). We performed a comprehensive histological analysis of all mammary glands and tumors at each NMU dose in an attempt to clarify what structural changes occur in the mammary gland before and after palpable tumors are evident. We observed a number of microscopic lesions and other epithelial abnormalities in the mammary glands for all NMU doses alongside the predicted increase in tumor burden and decrease in tumor latency with increasing NMU dose. Two types of non-neoplastic histological changes were observed in rats exposed to the 2 lower doses: (i) an increase in the number of acinar structures often accompanied by secretion into the lumen which is normally associated with pregnancy and lactation, and (ii) an increase in the number of epithelial cells sloughed into the lumen of the epithelial ducts. Our results indicate that this low-dose NMU carcinogenesis model provides a baseline for evaluating the relative susceptibility of animals protected from, or predisposed to, developing cancer when exposed to environmental toxins.

Below we address each reviewers concerns directly. Our comments are in boldface type. We wish to thank the Journal for the high caliber of Reviewers and in addition thank each Reviewer for their careful reading and thoughtful comments and suggestions. They were extremely helpful in creating this improved version of our manuscript.
Once again, we wish to assure the Editor that this manuscript is not being considered for publication elsewhere. It contains no copy written or personal communication material. All animals used in this research were treated humanely according to the institutional guidelines and all protocols were approved by the Institutional Animal Care and Use Committee. None of the authors have any professional or financial affiliations that could be perceived as influencing the work.

If you have any questions about the manuscript, please contact me at any time.

Sincerely,

Ana M. Soto, M.D.
Professor
Reviewer 1

Reviewer: Franca Formelli

Reviewer's report:
The authors accurately describe the relationship between the dose of NMU administered and the occurrence of neoplastic and non-neoplastic abnormalities in the mammary glands of Wistar-Furth rats.

Minor essential revisions.
1) The reported results clearly indicate that a true "non-carcinogenic" NMU dose is difficult to be defined. However, due to the range of dose investigated, the authors can only conclude that the lowest dose tested (10 mg/kg body weight) causes microscopic lesion and they cannot exclude that lower doses cause no histological changes.

Therefore the title and the abstract should be slightly modified. The title should describe the results and in the abstract, line 3: in order to ascertain whether a truly "non-carcinogenic" dose of NMU exists ...... at this aim, much lower doses should have been tested.
The authors agree and the abstract, title and text have been modified accordingly.

2) In the materials and methods section, the authors should specify that groups of 30-40 rats were used.
This has been done

3) In the results section, first paragraph, lines 8, 10: tumor number should replace tumor burden.
This has been done

4) Table 1 is confusing: the headings should be "all NMU doses" below 12 weeks and 22 weeks, "50 mg NMU/kg BW" below 25 weeks and "all NMU doses" below 30 weeks.
Table 1 was eliminated as requested by Reviewer 4

5) figure 1 and 2 legends: n= 29-40, /group should be added.
This has been done

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Reviewer 2

Reviewer: Nilda de Vargas Barbosa
Reviewer’s report:
Dear Editor,
This work proposed to conduct an NMU dose-response experiment for determining whether low doses of NMU are able in producing tumors or neoplastic alterations in mammary glands. The results obtained by group are a good contribution for development of experimental models of mammary carcinogenesis. The methodology used is sound, the manuscript is relatively well written and the work should be of interest to the general readership of the journal. However, I have some points that I realize that authors should consider to improve the quality of the manuscript. Below are some issues with the current draft.

Major Revisions
1. Title: the title must be more informative and to emphasize better the specific effects found with the low doses of NMU on mammary carcinogenesis in female Wistar-Furth rats. The title has been modified as per other reviewers.

2. All sections in the text must be numbered. This is not part of the formatting for BMC Cancer and was deemed unnecessary.

Introduction
1. It is not clearly explained in this section the significance to chemically-induced carcinogenesis studies offered by choosing of doses non-carcinogenic of NMU since this compound is firstly used to induce tumorogenesis process. The authors should incorporate this information arguing better the importance of search by the boundary between the non toxic and toxic activity of NMU. In my point of view, this information can be characterized as one of objectives of study. We thank the reviewer for the comments and we have modified the text of the Introduction to further clarify the objectives of this study.

2. A brief mention about the mechanism(s) of action(s) involved in the carcinogenic effect of NMU should be included in this section. This has been done.

Materials and Methods
1. Animal sub-item: I suggest that the sub-item “Tumor induction and detection” be added… This has been done

2. The authors should to specify how the latency and incidence measurements were recorded...for example: tumor incidence (% of animals that develop at least one tumor...??). This has been done
Results
1. What reasons the authors attribute to explain the differences found between thoracic and abdominal mammary glands with relation to latency and incidence parameters??

These features have been consistently documented in all susceptible strains of rats. However, to our knowledge, explanations for these events have not been provided so far, and we are unaware of the existence of credible data that would serve such purpose. We have combined the information regarding abdominal-inguinal and thoracic tumor latency as per Reviewer 4.

2. Incidence of palpable tumors Item: the last paragraph is confusing and needs to be rewritten
This has been done

3. The description of results related to histopathology of tumors is very extensive and can be condensed.

We thank the reviewer for her comments; however, the histopathology of the tumors and lesions is the focus of this paper. Therefore, we have decided to leave this section as written.

4. Page 10, last line: Adjust the sentence ...”time dependant”.. 
This has been done

5. Page 11, line 11: The term “dosed animals” should be substituted by groups (.. lower than in 20 and 30 NMU groups).

This has been done

6. Page 14, line 9: Replace the …“could be” by “were“

This has been done

Discussion and Conclusion
1. The language style of discussion must be improved.

We thank you for the comments. The discussion has been revised.

2. Page 17, paragraph 1: The last sentence ....."This is consistent with ......in neoplastic development”.... should be revised and rewritten

We respectfully submit that the Reviewer fails to clarify what it is that we should revise and how it should be rewritten. Therefore, the text remains as in the original manuscript.

3. Please edit the English of sentences below. It is not clear in present form. -“A dose-response study .................increased and the latency decreased as the dose was increased from 25mg NMU/kg BW to 75mg NMU/kg BW.” -“Similarly, an additional dose-response study highlights the importance of the route of NMU exposure as the Sprague-Dawley rats receiving an intravenous infusion of NMU
at 50 days of age seemed to develop tumors faster than those i.p. injected Wistar-Furth rats in our study; nevertheless, the overall tumor burden appeared similar.’

This has been done

4. The conclusion of work should be revised and rewritten too.
This has been done

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
Statistical review: No, the manuscript does not need to be seen by a statistician.

Reviewer 3
Reviewer: Denise Twinn
Reviewers report:
This study set out to define a dose response relationship for inducing mammary tumours in the Wistar-Furth inbred rat strain with the widely used carcinogen n-nitrosomethylurea (NMU). Virgin female rats of 49-58 d of age were injected with doses of 10, 20 30 and 50 mg/kg bodyweight NMU and the resulting palpable, and microscopic lesions were followed at 12, 22 and 25-30 weeks post-injection. An increase in tumour burden and decrease in latency was observed with increasing doses, but only for the higher doses of 30 and 50 mg/kg. 30 and 50 mg/kg doses of NMU resulted in both neoplastic and pre-neoplastic structures as early as 12 weeks post induction. Interestingly, preneoplastic aberrant structures were described and observed for the 2 lower doses (10 and 20 mg/kg bw) and these included a pregnant/lactational gland phenotype with milk production, sloughing of epithelial cells into the lumen, increased collagen expression in the stoma surrounding ducts, hyperplastic structures and DCIS structures. These by and large did not progress to palpable tumours within the time course studied. The authors suggest, as have others in the past, that tumour incidence and latency is strain dependent and propose that the lower doses may be used as a threshold model (described as “baseline” by the authors) for studying the impact of environmental or other additional insults for altering susceptibility.

The paper is well written and the question well defined. Methods are appropriate and well described. The data is sound and conclusions are balanced and supported by the data. The abstract is clear and concise and the title is appropriate. Similar studies have been carried out in the past, but this paper takes a further step in characterizing not only the effects of dose on latency and incidence but sets out to clearly define the properties of pre-neoplastic microscopic lesions.

Minor Revisions are recommended as follows-
1. Could authors comment on the apparent delay in incidence of inguinal palpable tumours occurring in 100% of animals given the 50mg dose compared to the thoracic tumours? (21w for inguinal vs. 16w for thoracic). **As it is not known why the thoracic tumors occur first and Reviewer 4 calls them the same biological entity, we have chosen to pool this data and not comment on it.**

2. It is worth mentioning the mechanism of action of NMU, i.e. its effects on DNA, why it targets mammary tissue and salivary tissue, but not other tissue types. **This has been done**

3. Could the authors comment on why the pre-neoplastic structures observed for the lower doses apparently do not progress to neoplasia? Would if therefore be more appropriate to call these lesions dysplastic rather that pre-neoplastic? **This has been done**

4. Mammary glands from one side were excise at 12w and the contralateral side at 22w. While this allows pairing of the data, the total incidence at each time point is being underestimated approximately by a factor of 2 (if we assume an approximately equal number of lesions to form on either side) compared to the final time point of 30w. **Thank you for the comment; we agree that this is a result of using paired data for the earlier timepoints and not the later ones.**

**Level of interest:** An article of importance in its field  
**Quality of written English:** Acceptable  
**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

Reviewer 4  
**Reviewer:** Tommaso Dragani  
**Reviewer’s report:**  
The manuscript of Murray et al., describes the results of a rat mammary carcinogenicity study with N-nitrosomethylurea (NMU). The authors have carried out a dose-response study using 4 doses of carcinogen: 10, 20, 30, and 50 mg/kg body weight (bw). The authors have carried out detailed analysis of gross and microscopic neoplastic lesions and concluded that “A true “non-carcinogenic” NMU dose may be difficult to define”. However, this statement is not based on their results. Indeed, (i) they have analyzed only a few doses of NMU and, most likely, the inclusion of doses below 5 mg/kg bw would have provided evidence of threshold; (ii) they have not carried out an adequate statistical analysis of the results.

As it has been pointed out in numerous publications, linear (arithmetic) scale for the dose of chemicals obscures effects at doses below those used in the experiment and distorts the effect seen over the range of doses used. It has been
proposed that, because of thermodynamic reasons, a logarithmic relationship of dose to biological effects must be used [Waddell WJ. Thermodynamic basis for expressing dose logarithmically. Toxicol Appl Pharmacol. 2008 Apr 15;228(2):156-7]. By such type of analysis, a threshold has been observed for many chemical carcinogens [Waddell WJ. Thresholds in chemical carcinogenesis: what are animal experiments telling us? Toxicol Pathol. 2003 May-Jun;31(3):260-2], and a threshold could also be observed by plotting the data of Table 3 of Murray et al., i.e., logarithm of the dose versus incidence of microscopic lesions. Such a threshold is estimated to be at just below 10 mg/kg bw NMU, and if the authors had carried out an experiment at 3 mg/kg bw NMU, most likely they would have observed a no-effect level of NMU on rat mammary carcinogenesis.

Therefore, the authors must quote the relevant papers of Waddell WJ and rewrite accordingly the Abstract, Discussion and Conclusion sections. Overall, this is an interesting manuscript that could be greatly improved by some relatively simple changes.

We have modified the text to reflect the fact that we were not looking for a threshold dose but rather characterizing low dose effects of NMU at the tissue level. We have rewritten the Abstract, Discussion and Conclusion and hope that this answers the concerns of this Reviewer.

Minor points:
1. Latency must be analyzed using time-based statistics that are used to test survival (e.g., Kaplan-Meier curves and log-rank test, Cox’s analysis). Analysis of tumor latency by ANOVA is a mistake. We thank the Reviewer for this comment. The latency analysis has been revised as suggested.

2. Tables 1 and 2 must be deleted and the relevant information should be moved to the Results. This has been done

3. Results of Table 3 at 25-30 weeks could be plotted in a nice dose-response graph, as I have drawn by reporting the dose in logarithm units (0 dose excluded) versus incidence of microscopic lesions, and adding a linear fitting of the data (r=0.94). As the focus of the paper is not on the identification of the threshold, we have chosen to leave the table as is and not include the graph as suggested here.

4. Tables 4 and 5 must be either deleted and their data briefly reported in the text, or summarized in a single Table reporting the dose-response overall data. This has been done

5. Figures 1B and 1C must be deleted since mammary tumors of thoracic or abdominal origin are the same biological entity.
This has been done

6. Figure 2 must become Figure 1B.
This has been done

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** Yes, and I have assessed the statistics in my report.