Author's response to reviews

Title: Dietary Flaxseed Administered Post Thoracic Radiation Treatment Improves Survival and Mitigates Radiation-Induced Pneumonopathy in Mice

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Author's response to reviews: see over
RE: Re-Revised manuscript submission (MS: 1709982096518081)

Dear Editor:

I am hereby submitting our re-revised manuscript entitled “Dietary Flaxseed Administered Post Thoracic Radiation Treatment Improves Survival and Mitigates Radiation-Induced Pneumonopathy In Mice” for publication at *BMC Cancer*. Both reviewer #1 and #2 had no concerns with the revised version of our paper and had expressed content with our response. Some minor formatting suggestions and a couple of grammatical errors have been addressed in this final version of the manuscript. Additionally, as suggested by the Editorial office Director, we have modified the front Page and the Figures per recommendations.

We are looking forward to a final acceptance of the revised manuscript,

With Best Regards,

Melpo Christofidou-Solomidou, Ph.D.
Referee#1: Reviewer's report:

COMMENT: This is an interesting study of C57BL/6 mice with 13.5 Gy thoracic irradiation with flaxseed dietary supplement. The authors look at 2, 4, 6 weeks after irradiation, measure fibrosis, and hydroxyproline content. The experimental model is not the model described by Travis for this mouse strain. Mice develop organizing alveolitis/fibrosis at 120 – 150 days after irradiation, and this is in fact organizing alveolitis not fibrosis. In addition, the irradiation dose of 13.5 Gy is below that usually associated with fibrosis in this model at 19 – 20 Gy. It is not clear what the investigators are measuring so that the results are going in a instructive direction. A critical control for the flaxseed has to be included.

RESPONSE: We have published extensively using this strain of mice and the dose of 13.5 Gy given as single fraction to the thorax. The C57/bl6 strain, is the “fibrosis prone” strain that has been used extensively by pioneering work of Franko and Sharplin (see Radiat Res. 1989 Oct;120(1):113-20; Radiat Res 1994, 140:347-355.). The dose of 13.5 Gy is within the range of the 14Gy used in numerous publications that helped establish the field and the parameters of lung damage/fibrosis set by these investigators. According to their findings, 14Gy single fraction to the lung induced “significant histologic lung fibrosis” and “radiation-induced hyaline membrane formation and focal fibrosis in the early phase (<28 weeks) of lung reaction”. Importantly, they report that “biochemical markers correlate with the histopathology” using hydroxy-proline content as a measure of lung fibrosis. Our study closely and measuring parameters follows that paradigm of radiation-induced lung injury and fibrosis.

We agree with the reviewer that including flaxseed is a critical control and we have done this (included in all figures). In fact we have FS as both non-irradiated and irradiated given preventively and compared to that given post-exposure.

Specific Comments:

Abstract:

COMMENT: The authors should include multiple radiation doses and data out to 120 – 150 days. The mouse strain used should be stated in the abstract.

RESPONSE: We stated the mouse strain in the abstract (see page 2).

We consider the suggestion to observe mice for 120-150 days (4-5 months) not far off from what we are currently using (4 months).

Regarding the suggestion to try yet another model of multiple, higher doses it is interesting but not necessary to prove the claims made in this study for the following reasons: We are confident that our radiation model (C57/BL6 mice; 13.5 Gy; 4 months observation) is an excellent model of radiation-induced lung fibrosis/inflammation/ survival and has provided very important information on radioprotection that we have published in numerous first tier, peer-reviewed journals:


One of the co-authors of this study is an experienced pathologist, Dr. Solomides, who has a 20-year career as a lung pathologist at academic institutions. He has confirmed the existence of pulmonary fibrosis and has described the Fibrotic Index in our publications as a semi-quantitative histopathologic assessment method of pneumonopathy, confirmed by quantitative biochemical assays as shown in all our studies, including the current.

**Introduction/Background:**

**COMMENT:** The discussion of radiation terrorism is inappropriate for the experiments being carried out with thoracic irradiation as the current model being studied is one more associated with radiotherapy for lung cancer or esophageal cancer. The introduction discusses cytokine radioprotective drugs, which are irrelevant to the current study and should be deleted.

**RESPONSE:** We respectfully disagree with the reviewer with the comment that discussion of radiation terrorism is not relevant since what we are using is not a model of radioprotection but of mitigation of adverse toxicities associated with exposure. We are interfering with the toxicity after it has been established, i.e., post-exposure.

The mention of radioprotective drugs (not only of cytokine but of receptor blockers etc) in the introduction is only to make the point that current radioprotectors are being evaluated as mitigators with limited success.

**Materials and Methods:**

**COMMENT:** C57BL/6J mice from Charles River should be described in greater detail. C57BL/6TAC compared to C57BL/6HnSD have different radiobiology and have been published by others, and the Charles Rivers strain should be discussed with respect to references 18 – 20. They have used different mice.

**RESPONSE:** We have responded above regarding the use of the mouse strain and the radiation dose. The radiobiology of this strain has been extensively described in our previous publications listed above.

**COMMENT:** A complete dose response curve for induction of radiation fibrosis in these mice at 120 – 150 days should be included in preliminary data or in a revised manuscript since this is critical to interpreting the current dietary supplement.

**RESPONSE:** See previous response above regarding time/dose selected. We have provided references to our earlier publications regarding radiation details such as shielding, etc. We have 3 ways of establishing fibrosis, a qualitative, a semiquantitative, and a quantitative assay, including the Fibrotic Index which we have published and described extensively.
**COMMENT:** Dietary treatments should include a control FS.

**RESPONSE:** An isocaloric irradiated and non-irradiated control FS group is indeed included among the groups. In addition we included an non-irradiated FS-supplemented control group. In fact, all statistical comparisons are made between the non-irradiated, control FS group and all the irradiated, FS groups.

**COMMENT:** 13.5 Gy as described with 250 KVP should be related upon. What was the dose rate? What was the shielding applied? What was the dose to the head, and abdomen/pelvis?

**RESPONSE:** Although described in our previous publications, we have added information regarding the irradiation procedure (see page 7).
Specifically we state: “Mice were anesthetized and irradiated as previously described [10]. Briefly, using a customized immobilization chamber that allows bilateral exposure of the lung of up to 8 mice simultaneously while lead shielding (3mm) the head, abdomen and extremities, 13.5 Gy was delivered to mid-plane using a 250kVp orthovoltage machine (Philips RT 250) at a dose/rate of 1.7 Gy/min and a source to skin distance of 33 cm, through a 0.2mm copper filter and a tube current of 13mA. For quality assurance, thermoluminescent dosimeters were placed over selected mice to verify correct dose administration.”

**COMMENT:** The other sections of the Materials and Methods section are appropriate except for the tissue harvesting and evaluation. The Optimus Imaging System should be utilized to quantitate percent organizing alveolitis/fibrosis as described by Travis.

**RESPONSE:** The suggestion to use an Imaging system to quantify fibrosis is redundant. As explained above, we have 3 ways of indicating fibrosis in our manuscript. In addition, the particular system suggested is not widely used. We have searched extensively PubMed and found no articles using this system.

**COMMENT:** Hydroxyproline content is interesting but must be accompanied by data at 120 – 150 days with respect to organizing alveolitis/fibrosis.

**RESPONSE:** We find it alarming that the reviewer finds the established assay of measuring tissue levels of hydroxyproline as a measure of fibrosis as “interesting”. We searched PubMed using the terms “hydroxyproline” and “fibrosis” and came up with 1961 published articles! In lung alone, there are 816 papers that use this parameter to describe their findings. So, use of this methodology to quantify fibrotic tissue changes is well established and widely accepted in the field. We have been extensively using this methodology in our peer reviewed publications in conjunction with several additional measurements to describe fibrotic tissue changes as confirmed histologically by an experienced lung pathologist.

**Results:**

**COMMENT:** Table 1 describes data with BAL at 4 months which would be 16 weeks for 112 days. Data at 150 days should be included to measure organizing alveolitis as described by Travis. Multiple irradiation doses should be tested including 19 Gy and 20 Gy previously reported by Travis to produce fibrosis/organizing alveolitis in this strain.

**RESPONSE:** We have addressed above this issue of timing and fibrotic changes. At 4 months post XRT and with the established conditions of the experiment, we have florid inflammation and established fibrotic changes. The selected dose is justified by studies performed by other investigators and our group and we have described this in the manuscript.
The reviewer maintains that we are not producing interstitial fibrosis in our model. As we clearly show in Fig.9B, our radiated lungs evidence diffuse interstitial collagen upon staining with the Masson Trichrome stain, a feature not seen in the controls Fig.9A. That this staining pattern is indeed interstitial collagen was confirmed by the increased hydroxyproline content of these lungs in comparison again with the control.

**COMMENT:** Figure 1 is a paradigm and could be described in the text.
**RESPONSE:** We have indeed described this in the first paragraph of the Results section.

**COMMENT:** Figure 2 describes FS compared to control diet. The results are quite interesting out to 6 weeks, but the data for 16 weeks, and more appropriately 150 days should also be included. One of the problems with studies of late fibrosis in this mouse strain is the failure to hold mice long enough to detect fibrosis/organizing alveolitis.
**RESPONSE:** We have addressed the issue of the model used, earlier in our response.

**COMMENT:** Figure 3 describes effects on body weight showing minimal differences. This is instructive as it suggests that the animals are eating the diet.
**RESPONSE:** This figure shows more than just the fact that “mice are eating the diet”. It shows that the significant drop in body weight in mice on control diet is mitigated by the addition of flaxseed in the diet.

**COMMENT:** Figure 4 shows data out to 6 weeks. The study has suggested rapid death at 110 and 150 days which is just the time that these experiments were terminated for the diet group. Groups should be included in which the mice were fed FS for the entire 150 days. Panel B shows minimal changes. It is not clear how the data in panel A (supplementary), on BAL WBC/ml relates to the other studies of cytokines and fibrosis.
**RESPONSE:** We have clarified in the text that the FS group was indeed fed the diet for the entire duration of the experiment as the reviewer suggested.

**COMMENT:** Figure 5 describing lung injury, inflammation, and blood oxygenation are not particularly informative. Panel B shows thickening, but there is no specific stain for fibrosis rather HE. This could be an acute reaction and does not look like the fibrosis/organizing alveolitis seen in this mouse strain at 110 – 150 days post-thoracic irradiation.
**RESPONSE:** We find these comments confusing. Figure 5 is on injury, inflammation and blood oxygenation and should be one of the most informative slides since these are parameters extremely relevant to the radiation lung toxicity and most accurately reflect the benefit of the diet supplied. The comment on Panel B, is confusing since we think it refers to Figure 6B which indeed it is an H&E stain only because it shows the proteinaceous exudate reflective of the lung edema/injury. The suggestion to show a specific stain for fibrosis is good, and indeed it is the focus of the entire Figure 9! That entire figure shows Mason’s blue, Trichrome staining, namely staining for collagen and fibrotic changes. It is rightfully placed thematically along with the fibrosis measurement slides.

**COMMENT:** Figure 7 shows lipid peroxidation that appears to be decreased by the short term administration of FS. This data is quite interesting.
**RESPONSE:** We agree that decreased oxidative tissue changes are very important and worthy of indicating.
**COMMENT:** Figure 8 fibrotic changes in mouse lungs and fibrotic index should be accompanied by Optimus Imaging. This mouse strain gets peripheral lung organizing alveolitis rather than diffuse fibrosis and would be expected to be seen in the photographs. The results and discussion section follows the figures and tables.

**RESPONSE:** We have addressed this above.

**Discussion:**

**COMMENT:** The discussion should include mention of the new data requested, and more importantly, an analysis of fibrosis/organizing alveolitis in this mouse strain and how FS might influence the molecular pathways thought to be associated with lung lesion as described by Travis.

**RESPONSE:** We have addressed this issue above.

**Level of interest:**

**COMMENT:** An article whose findings are important to those with closely related research interests

**Quality of written English:**

**COMMENT:** Not suitable for publication unless extensively edited

**RESPONSE:** We have revised and extensively edited the manuscript for language.

**Statistical review:**

**COMMENT:** No, the manuscript does not need to be seen by a statistician.

**RESPONSE:** We have used statisticians in analyzing the data.

**Declaration of competing interests:**

**COMMENT:** I declare that I have no competing interests below

**RESPONSE:** We have evidence to suggest conflict of interest for this reviewer:


**Referee#2:**

**Reviewer's report:**

**COMMENT:** This manuscript evaluates the efficacy of a flax seed diet in the mitigation of radiation-induced lung sequelae. This is a well-executed study that should prove important for improving treatment of radiation injury in the lungs, and possibly in general, not only in the event of a nuclear accident or terrorist attack but also during radiation therapy. The study continues from the author’s previous work on dietary flax seed’s ability to protect lung when administered before irradiation. In this manuscript, they show a remarkable effect of dietary supplementation after irradiation on inflammatory response in the lung. Overall, there are no major problems.

**RESPONSE:** We are thankful for the comment.

The following are some minor questions that need to be addressed:

**Minor Essential Revisions**
COMMENT: 1) Should mention in figure 1 legend and materials and methods page 7 that the mice were maintained on the diet after initiation until termination. Page 12, first paragraph; it needs to be clarified whether the mice were maintained on their respective diets for 4 months or each group was on the diet for 19, 16, 14, 12, or 10 weeks – the latter appears to be the case (according to figure 1), but the text seems to imply the former. It becomes clear after reading the entire article, but there is ambiguity in the beginning, which needs to be corrected.
RESPONSE: We have made all suggested changes (see page 7)

COMMENT: 2) What is the HVL of the x-rays? Or if the HVL is unknown, give the manufacturer of the x-ray tube.
RESPONSE: We have made all suggested changes/additions-see page 7/8.

COMMENT: 3) In the survival plot, ‘n’ is given as 20 and 30 for unirradiated and irradiated groups, respectively. Does this mean 10 mice each for the 0% FS and 10%FS groups and 5 mice each for the irradiated groups? Was this repeated for the second independent experiment so that actually there were 40 and 60 mice used total?
RESPONSE: We apologize for the lack of clarity. We clarified in the text the “n” for each cohort as suggested. The given numbers actually reflect combined data from 2 independent identical studies. We have made all suggested changes.

COMMENT: 4) There are misspellings and grammatical mistakes throughout the manuscript. Some, but not all, have been listed here: Page 4, incomplete last sentence of first paragraph; Page 10, line 5, “pre-adopted” should be “pre-adapted”; Page 12, top, it should just be “results” not “results and discussion”; Page 12, 4 lines from bottom “let” should be “led”; Figure 2 legend, last line should be deleted; Figure 3, the dotted line described in the legend is missing; Page 17, line 1, “o significant” should be “a significant”; Page 21, first line, second paragraph “leeds” should be “leads”...
RESPONSE: We have made all suggested changes. We apologize for all the grammatical errors. We have extensively edited the manuscript for language.

Discretionary Revisions
COMMENT: 1) What is the significance of ED versus EL levels in the plasma? It is interesting that the EL/ED ratio increases in the FS+IR (0 wk) and FS+IR (+6 wks). Is there any radiation-induced metabolic significance to this? Certainly others have shown that the anti-oxidative potency of EL and ED are different; interestingly, although ED may be a stronger antioxidant, EL appears to be more effective against tumor cell lines. Additionally, regardless of the ratio, it is clear that irradiation decreases plasma ED levels and increases EL levels.
RESPONSE: We showed this data as an indication that a) mice consume the diet; b) the thoracic radiation has not affected the gut (we used lead shielding of the abdomen) so metabolic conversion of the lignan precursor SDG can be made to the mammalian lignans ED/EL by the gut flora. We are not confident to make any assessment as to the meaning of the ED/EL levels in the circulation. Additional studies will be needed to derive any conclusions as to the significance of one lignan versus the other.

COMMENT: 2) Change in body weight as a function of time would be much more informative than final body weight at the end of the study, since mice are continually dying throughout the 4 months and the thoracic irradiation will probably cause transient changes in weight as waves in cellular responses come and go during the 16 weeks, which may be modified by the diet as well.
**RESPONSE:** We agree with the comment and we will monitor weight longitudinally in future studies.

**COMMENT:** 3) Considering all the time and resources used to measure numerous cytokines, a more extensive discussion and interpretation of the results would be helpful.

**RESPONSE:** We agree with the reviewer. We initially had an extensive discussion on the cytokines in our manuscript which we decided to remove from the submitted manuscript due to space restrictions. We added an abbreviated version of that text back into the discussion (see page 21/22). We included an additional reference. We abbreviated the materials and methods section to accommodate a longer Discussion section.

**Level of interest:**

**COMMENT:** An article of outstanding merit and interest in its field

**RESPONSE:** We are thankful for the comment.

**Quality of written English:**

**COMMENT:** Needs some language corrections before being published

**RESPONSE:** We have made extensive editing for language.

**Statistical review:**

**COMMENT:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

**COMMENT:** I declare that I have no competing interests.