

Dear Editor of Antioxidants,

I send herewith, our revised manuscript (Manuscript ID: antioxidants-1025902), entitled "

Effect of rosmarinic acid and ionizing radiation on glutathione in melanoma B16F10 cells: a translational opportunity"" with the major revisions and changes suggested by the referees.

Following the instructions of the Assistant Editor, we confirm the selection of "Open Review" for our manuscript and have added the Conclusion section.

We thank the reviewers for their efforts in correcting and improving the paper.

I hope that these changes will be found acceptable and patiently await your response/decision.

Sincerely,

Miguel Alcaraz

Reviewer #1: Manuscript ID: antioxidants-1025902

Comment. This study investigated the effect of rosmarinic acid (RA) on the melanoma B16F10 cells versus human prostate epithelial cells (PNT2) in the GSH intracellular production. This study has done a very simple study and observed the results of a simple phenomenon. The study for the intracellular mechanism on RA is also not investigated.

I have some comments and questions as follows:

1. You should study antioxidant defense via direct interaction with ROS or via activities of detoxication enzymes like SOD and glutathione peroxidases (GPx).

Response. Evidence from Radiotherapy and Radiobiology reveals that, cellular exposure to a high dose of ionizing radiation leads to an ionizing radiation induced oxidative stress that produces an increase in oxidized glutathione and a decrease in reduced glutathione, with the consequent decrease in GSH / GSSH ratio, accompanied by depletion of total intracellular glutathione.

However, our results showed the opposite during the first hours after irradiation: we determined a decrease in oxidized glutathione, and an increase in reduced glutathione levels, which led to an increase in the GSH / GSSH ratio in some cells and subsequently an increase the amount of total intracellular glutathione levels.

The determinations were repeated several times and the technique modified as recommended by the manufacturer (eliminating dead cells, filtering the culture media), but the same results were obtained.

We consulted with an international specialist in glutathione and melanoma studies (Prof. Francisco Solano, Prof. Biochemistry and Molecular, references 23 and 24), to find a possible explanation for this discrepancy of our findings with previously reported results. We have thus far considered our results as an initial cellular defensive response, although some authors have considered it an adaptive response to low doses of ionizing radiation.

Under these circumstances, we choose to present the methodology and results of our work in a very simple manner making reproducibility of our work by peers a possibility in order that discrepancies, of great clinical interest, can be adequately assessed by specialists. We are currently studying antioxidant defenses via direct interaction with ROS which, even though our are data preliminary, seem to confirm our results.

we incorporated the following paragraph in the text of the manuscript (line 391):

"Obviously more studies are necessary to confirm our results that will allow us to analyze the antioxidant defense response through interactions with radioinduced ROS (DPPH assay, Ferric reducing antioxidant power assay, Nitro blue tetrazolium reduction assay, ROS assay), and through

the evaluation of detoxifying abilities of enzymes like SOD and glutathione peroxidases (GPx)"

Comment. 2. You should change English

Line 23, this type of substances --> this type of substance

Line 24, provide protection to --> protect

Line 36, radiosensitizing agent --> a radiosensitizing agent

Line 45, mechanisms affects --> mechanisms affect

Line 51, could provide protection to healthy --> could protect healthy

Line 58, Bovine serum albumen --> Bovine serum albumin

Line 94, was confirmed by means of thermoluminescent --> was confirmed utilizing thermoluminescent

Response. -All of these changes have been made.

Reviewer 2

Comment. The present work by Olivares et al looks at the effects of Rosmarinic acid (RA) on an immortalized human prostate epithelial cell line (PNT2) and murine melanoma cells (B16F10) following exposure to high dose (20Gy) X-rays. The authors measure reduced glutathione at early timepoints (1, 3h) as a proxy for free radicals/oxidative stress. The authors argue that RA is protective in the immortalized cell line following high-dose irradiation which would argue for application of this drug concomitantly with irradiation of tumours to protect neighbouring, non-cancerous tissues. The manuscript is well-written and to the point. Notwithstanding minor issues concerning presentation of the data/statistics, I do have a major specificity concern which prevents me from recommending publication unless it can be adequately addressed:

In Reference 11 (Alvarez et al, 2014), the authors used the same cell models to address the effects of RA on cell viability following X-Ray irradiation. There was a large effect of DMSO on radiosensitivity (10Gy) in the same PNT2 cell line (Figure 4) which seemed to be radioprotective at 24 and 48 hr. In fact, DMSO appeared to be more radioprotective than RA. DMSO is a known free radical scavenger and as such is radio-protective in and of itself. In the present study it wasn't clear whether the authors used vehicle controls. Was RA dissolved in DMSO, and if so were controls treated with a similar volume of DMSO? If not, these experiments would need to be repeated to discount any effects of the DMSO vehicle on the radiation-mediated changes in glutathione levels. Experiments using another vehicle or an experiment showing a dose-response effect of RA are required. Thus, the authors' assertion that RA has a specific, radioprotective effect cannot be adequately evaluated in the context of the current work as presented.

Response:

- In PNT2 cultures we used RPMI culture medium and DMEM culture medium for B16F10 cells, in both cases, media were supplemented with 10% fetal bovine serum, but in neither culture was DMSO added. DMSO was only used during the cell lysing protocol in the MTT assay and during cryopreservation. Never in the culture medium has DMSO been used for cell growth.
- On the other hand, RA is water soluble and was administered dissolved in PBS, and was not on any occasion dissolved in DMSO.

As our referee has commented, DMSO is a powerful radioprotectant which we also previously studied with these two cell

lines. The following graphs show the results obtained with DMSO under the same experimental conditions and doses similar to the treatments administered in reference 11 (results not published until now):

PNT2 and DMSO

After 24 hours of incubation the results obtained from PNT2 cell cultures with the two concentrations of DMSO (0.1% and 0.2%) showed higher cell survival with respect to the irradiated control cells. While a 30.8% reduction in cell proliferation was observed for the irradiated control cultures at the highest dose employed, a 22.3% reduction in cell proliferation was obtained when cells were exposed to 0.2% DMSO. When cells were exposed to 0.1% DMSO and irradiated at 10 Gy, no reduction in cell proliferation was noticed. At both concentrations of DMSO studied, an increase in cell survival was observed compared with irradiated control cell cultures ($p < 0.001$) (Figure M8). The Protection Factor (PF) obtained for cultures treated with 0.1% DMSO following 24 hour incubation and exposure to the with 10 Gy of X rays was 100% and 47% when 0.2% DMSO solution was used at the same radiation dose.

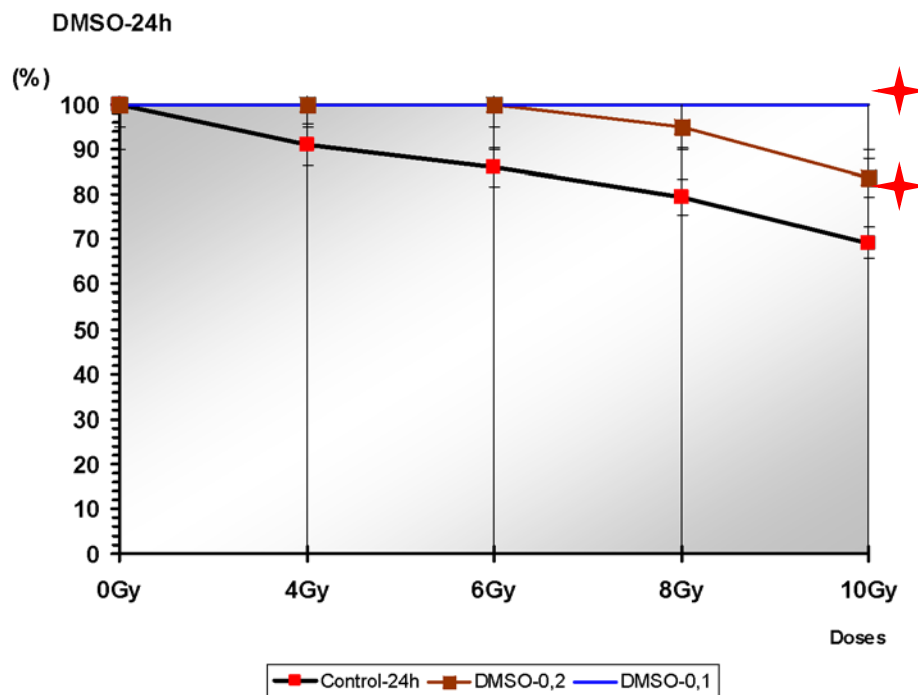


Figure M8. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 24 hours of incubation (*): ($p < 0.001$) versus irradiated control cells.

After 48 hours of incubation, the cell survival results obtained for the PNT2 cell cultures treated with the two levels of DMSO (0.1% and 0.2%) were equal but higher than that in the irradiated control group. Both concentrations produced the same effect of 38.8% increase in cell survival numbers compared with the irradiated controls. At both concentrations of DMSO employed, an increase in cellular survival was shown compared with irradiated control cells ($p < 0.001$) (Figure M9). The PF obtained with cell cultures treated with the two concentrations of DMSO for 48 hours after exposure to 10 Gy of X-rays was 100% for both 0.1% and 0.2% DMSO.

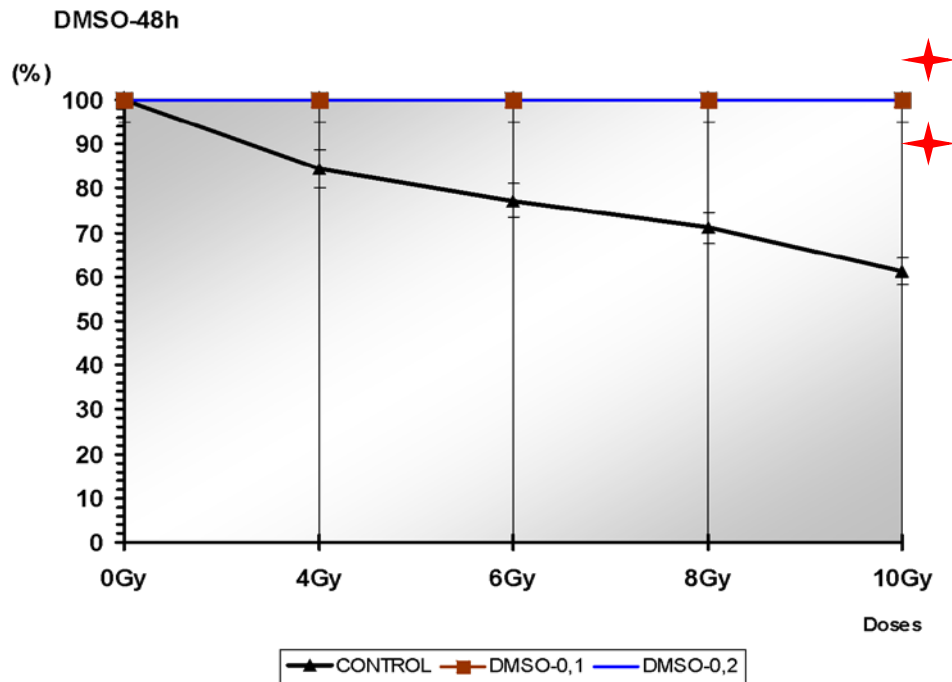


Figure M9. Cell survival curves of normal human prostate epithelial PNT2 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 48 hours of incubation (*): ($p < 0.001$) versus irradiated control cells.

B16F10 and DMSO

After 24 hours incubation of the B16F10 cells with the two concentrations of DMSO studied (i.e., 0.1% and 0.2%) and exposure to 10 Gy, a higher cell survival of 21.8 % with respect to irradiated control cells was obtained for the lower DMSO concentration tested (0.1%) while a less superior cell survival of 10.1% with respect to irradiated control cultures was obtained for the higher DMSO concentration (0.2%). At both concentrations of DMSO studied, a significant increase in the survival of B16F10 melanoma cells was established when compared with irradiated control cell cultures ($p < 0.01$) (Figure M34). The radiation Protection Factor (PF) obtained from 24 hour cultures of B16F10 cells conditioned with 0.1% DMSO and exposed to 10 Gy of radiation was 65.8% while a PF of 30.5 % was established for cultures treated with the higher DMSO concentration (0.2%) and the same radiation dose and incubation period.

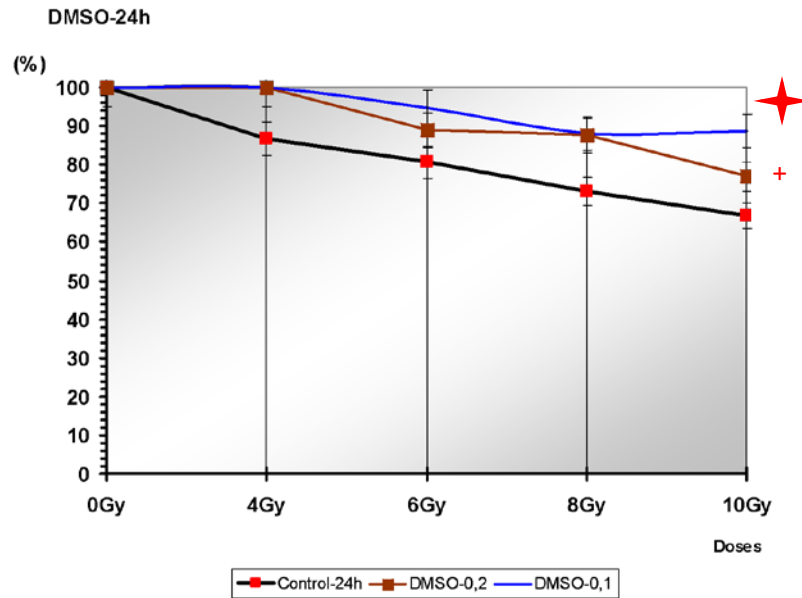


Figure M34. Cell survival curves of B16F10 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 24 hours of incubation (+): ($p < 0.01$) versus irradiated control cell cultures; (*): ($p < 0.001$) versus irradiated control cell cultures).

After 48 hours of incubation following exposure of B16F10 cells to both concentrations of DMSO (0.1% and 0.2%) assayed, a higher cell survival of 6.1% relative to irradiated controls was established with the 0.1% DMSO solution at a radiation dose of 10 Gy, while the higher concentration of DMSO (0.2%) produced a cell survival of 7% less than the irradiated controls at 10 Gy. After 48h of incubation, the cell survival values obtained for B16F10 melanoma cells cultures treated with two levels of DMSO (0.1% and 0.2%) did not show significant differences compared with the cell survival value obtained with irradiated control cell cultures (Figure M35). Protection Factor (PF) obtained for 48 hour B16F10 cell cultures conditioned with 0.1% DMSO and exposed to 10 Gy was 14.8% and zero (i.e., a lack of protective capacity) when 0.2 % DMSO was used.

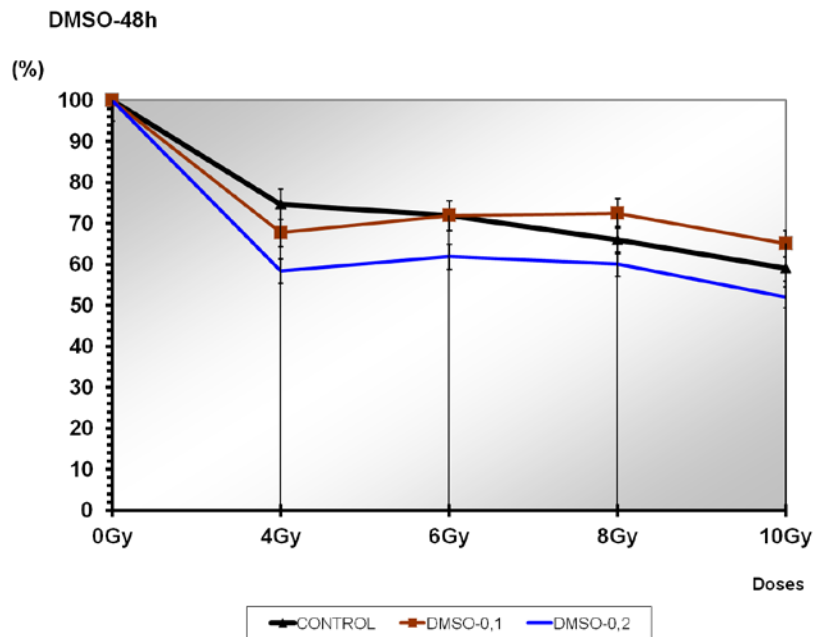


Figure M35. Cell survival curves of B16F10 cell cultures treated with 0.1% and 0.2% of DMSO and exposed to different doses of X-rays evaluated after 48 hours of incubation.

(To determine the protection factor (PF), the formula for the factor protection (reference 11) modified for the analysis of growth inhibition at 10 Gy and incubated 48 h was used: $PF(\%) = [(MT - MC) / MC] \times 100$, where MT is the mortality of the cells irradiated and treated with the substances studied; and MC is the mortality of the irradiated control cells).

Reviewer 3

Comment. This manuscript studies showed that the efficacy of rosmarinic acid is due to glutathione in cells. First, the author must explain the difference from the previous study such as below.

“ Berivan Tandogan et al., In vitro effects of rosmarinic acid on glutathione reductase and glucose 6-phosphate dehydrogenase. Pharm Biol. 2011 Jun;49(6):587-94. “

Response. In our opinion, our study continues from the end of the work of Tandogan and co-workers (reference 32 in our manuscript). Tandogan et al. describe the in vitro effect of RA under normal or physiological conditions. Thus, they demonstrate that under in vitro conditions, cells of the brain, kidney and liver, RA inhibits the enzymes glutathione reductase and glucose 6-phosphate dehydrogenase in a concentration dependent manner. They highlight that, although RA has antioxidant properties, high concentrations of RA inhibit both G6PD and GR activity. GR inhibition may influence total intracellular GSH levels and lower cellular defense against oxidative stress. They indicate that the mechanism of RA inhibition may be due to the interaction of RA with sensitive SH groups present in G6PD. G6PD activity is thought to depend on the intactness of SH groups; inhibitory effect of RA may be due to binding thiol groups in the enzyme. In consideration of the amino acid residue in the substrate and NADP⁺-binding site or catalytic site, cysteine residues were found, which play an essential role for the activation of G6PD. Finally, they suggest that because G6PD is an important enzyme in many reductive biosynthesis and GSH regeneration, its inhibition by RA may induce oxidative damage that causes hemolytic anemia in G6PD-deficient individuals.

Based on interest in the response of cancer patients with cells with two different degrees of radiosensitivity to radiation, we induced a pathological condition (exposure to high doses of ionizing radiation) and determined two

responses that are opposed to each other and found even surprising responses when contrasted with the report of Tandogan et al.:

- Under normal, **physiological conditions**, in prostate and melanoma cells, the administration of RA produces a decrease in the GSH / GSSG ratio (Fig. 4b) and total glutathione levels in consonance with the report of Tandogan et al.

- However, in the **pathological conditions** that we induced, which are the object of our study, two opposing responses are likely to be produced:

A) In prostate cells treated with RA and radiation, we should have determined a decrease in GSH concentration and the GSH/GSSH ratio due to the inhibition of the enzymes glutathione reductase and glucose 6-phosphate dehydrogenase; inhibition that would exceed the antioxidant capacity of RA and thus showing greater oxidative damage in cells as described by Tandogan et al. However, in our study we determined, in prostate cells treated with RA and radiation, a significant increase in the GSH / GSSG ratio (Fig. 4c) with a normal glutathione concentration (Fig. 3) that would reveal a synergistic effect of RA as an antioxidant that would add to the effect of endogenous GSH. Furthermore, the consumption of RA by radio-induced free radicals would produce less inhibition of antioxidant enzymes (Fig. 4c and 4d), thus protecting the prostate cells; however, we found the greatest ionizing radiation induced cellular damage (oxidative stress) in these cells.

B) In the metastatic B16F10 cells treated with RA and radiation, we should have determined a decrease in GSH concentration and GSH / GSSG ratio due to the inhibition of the enzymes glutathione reductase and glucose 6-phosphate dehydrogenase; an inhibition that would exceed the antioxidant capacity of RA and would thus show greater oxidative damage in cells as described by Tandogan et al. Now, in our study, in the B16F10 melanoma cells treated with RA and radiation, we found a no variations in the total GSH concentration (Fig. 3) with an increase in the GSH / GSSG ratio compared to non-irradiated control cells (Fig. D), which could not justify the enormous oxidative damage (greater radiosensitivity) in these melanoma cells as described by Tandogan et al., nor the significant decrease in the GSH / GSSG ratio (Fig. 5) compared to irradiated melanoma cells without RA treatment.

The objective of our study was not to determine the effect of RA on cells (prostate or melanoma) under physiological conditions, but on the cellular response under conditions of oxidative stress, since it could allow the protection of very radiosensitive cells normal cells with RA and at the same time cause more damage to the radio-resistant cells (tumor cells) if both are exposed to the same irradiation field as in cancer treatment

Comment. Overall, the following additional data is required:

1. I don't understand the need for Fig2 and meaning for Fig3b.

Response. - **Fig2:** (we replicate a few paragraphs in response to reviewer 1 to answer this comment):

Evidence from Radiotherapy and Radiobiology reveals that, cellular exposure to a high dose of ionizing radiation leads to an ionizing radiation induced oxidative stress that produces an increase in oxidized glutathione and a decrease in reduced glutathione, with the consequent decrease in GSH / GSSH ratio, accompanied by depletion of total intracellular glutathione.

However, our results showed the opposite during the first hours after irradiation: we determined a decrease in oxidized glutathione, and an increase in reduced glutathione levels, which led to an increase in the GSH / GSSH ratio in some cells and subsequently an increase the amount of total intracellular glutathione levels. The determinations were repeated several times and the technique modified as recommended by the manufacturer (eliminating dead cells, filtering the culture media), but the same results were obtained. We consulted with an international specialist in glutathione and melanoma studies (Prof. Francisco Solano, Prof. Biochemistry and Molecular, references 23 and 24), to find a possible explanation for this discrepancy of our findings with previously reported results. We have thus far considered our results as an initial cellular defensive response, although some authors have considered it an adaptive response to low doses of ionizing radiation.

Under these circumstances, we choose to present the methodology and results of our work in a very simple manner making reproducibility of our work by peers a possibility in order that discrepancies, of great clinical interest, can be adequately assessed by specialists.

Given that we used an easily acquired commercial test, the greatest criticism that we have considered that could be made to our study is an inadequate use of the test and using an inadequate standard glutathione curve

For this reason, we have included Fig. 2, even though our Glutathione standard curve is not identical to the one presented by the manufacturer, given that the results obtained does not exceed 500,000 RLUs, it lies within the linear area of the dose-response curve, presenting a suitable slope and reproducibility for the study.

If reviewer 3 considers that these circumstances do not justify the incorporation of Fig. 2 into the manuscript, we have no problem in deleting it.

-Fig3b:

In radiobiology, it has traditionally been considered that the amount of total cellular glutathione and capacity to generate GSH in a situation of oxidative stress are the factors that influence the radio resistance of a cell. Melanoma cells are classically the model of radioresistant cancer cells, as they have a very high amount of total glutathione and an enormous capacity to generate endogenous glutathione in situations of oxidative stress (Fig. 2a).

However, we have not found any reference in the scientific literature that describes that traditionally radiosensitive cells (such as those of the prostate) are capable of rapidly generating an amount of endogenous glutathione significantly greater than the metastatic melanoma cells themselves ($p < 0.001$), in percentage terms, more than 30% of the amounts determined in B16F10 cells, and almost doubling their total GSH content when exposed to ionizing radiation (Fig. 3b).

If the reviewer 3 considers that these circumstances do not justify the incorporation of Fig. 3b into the manuscript, we have no problem in omitting it.

Comment. 1. Ascorbic acid should be put into the data as a control group.

2. Various analyses of antioxidants are required. Ex) DPPH assay, Ferric reducing antioxidant power assay, Nitro blue tetrazolium reduction assay, ROS assay

Response. Thank you very much for your comments. We will implement your suggestions in our next analyzes to confirm these results. As a limitation of the study we have included the following paragraph (line 391):

"Obviously, more studies are necessary to confirm our results and to better analyze the antioxidant defense response and its interaction with radioinduced ROS (using DPPH assay, Ferric reducing antioxidant power assay, Nitro blue tetrazolium reduction assay, or ROS assay), and also by determining the capacity of detoxifying enzymes like SOD and glutathione peroxidases (GPx)".

Comment. -It is necessary to present mechanisms for rosmarinic acid and glutathione

Ex) Caroline Gaucher et al., Glutathione: Antioxidant Properties Dedicated to Nanotechnologies. Antioxidants (Basel). 2018 May; 7(5): 62.

Montserrat Mari et al, Mitochondrial Glutathione, a Key Survival Antioxidant. Antioxid Redox Signal. 2009 Nov; 11(11): 2685–2700.

Response. In presenting the possible mechanisms by which RA can modify the generation and concentration of intracellular glutathione, we can consider two different situations:

a) In unirradiated normal cells treated with RA, considered as controls and under physiological conditions, we incorporated the following paragraph that would present a possible mechanism described by Tandogan et al. (line 250):

"We found no references on the effect of RA on intracellular GSH in the PNT2 cells. In brain, kidney and liver cells treated in vitro with high concentrations of RA, the inhibition of the enzymes glutathione reductase (GR) and glucose 6-phosphate dehydrogenase (G6PD) have been described, highlighting that, although RA has antioxidant properties, high concentrations of RA inhibit both G6PD and GR activity. GR inhibition may influence total intracellular GSH levels and lowers cellular defense against oxidative stress. They indicate that the mechanism RA inhibition may be due to the interaction of RA with sensitive SH groups present in G6PD. G6PD activity is thought to depend on the intactness of SH groups; inhibitory effect of RA may be due to binding thiol groups in the enzyme. In consideration of the amino acid residue in the substrate and NADP+-

binding site or catalytic site, cysteine residues were found, which play an essential role for the activation of G6PD. Finally, they suggest that because G6PD is an important enzyme in many reductive biosynthesis and GSH regeneration, its inhibition by RA may induce oxidative damage that causes hemolytic anemia in G6PD-deficient individuals (32)".

- b) In irradiated cells treated with RA, which is the object of our work, we have not been able to find a mechanism other than the antioxidant capacity of RA in PNT2 cells. In melanoma cells, where the most important aspect of the study lies due to its clinical significance, we have suggested a new mechanism (lines 302-389)

We have incorporated the new references into the bibliography section:

- Mar, M.; Morales, A.; Colell, A.; García-Ruiz, C.; Fernández-Checa, J.C. Mitochondrial glutathione, a key survival antioxidant. *Antioxid Redox Signal* **2009**, 11, 2685-2700. DOI: 10.1089/ARS.2009.2695
- Gaucher, C.; Boudier, A.; Bonetti, J.; Clarot, I.; Leroy, P.; Parent, M. Glutathione: Antioxidant Properties Dedicated to Nanotechnologies. *Antioxidants* **2018**, 7(5):62. DOI: 10.3390/antiox7050062.

We thank the referees for their contributions and the effort made that has allowed us to significantly improve the text presented.