

To Prof. Dr. Maria Luz Fernandez
Editor-in-Chief
Nutrients

Answers to Reviewers

Manuscript ID: nutrients-2998799

We are very grateful to the reviewers for their appropriate and constructive suggestions. The suggestions raised by the reviewers were all pertinent and we agree entirely with their observations. To comply with their requests, we have made some changes in the original version of the manuscript.

We believe that these changes very improved the quality of the manuscript.

The reviewers' requests are highlighted in "red" in the new version of the manuscript; however, other changes were made throughout the manuscript attempting to make the text in an intelligible way. Extensive revision of the English grammar was performed, in all sections of the manuscript.

We have answered all the comments of the reviewer individually, as follows:

- ❖ **Reviewer #1:** Comments 1 to 7
 Comments about Tables 1 and 2
 Comments about Figures 2-8

- ❖ **Reviewer #2:** Comments and Suggestions for Authors

Thank you very much for your kind consideration of this resubmitted version of our manuscript.

Sincerely,

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❖ Response to Reviewer #1

Comments and Suggestions for Authors

General comments

The manuscript entitled “Lycopene in combination with insulin triggers antioxidant defenses and increases the expression of components that detoxify advanced glycation products in kidneys of diabetic rats” by Ingrid Delbone Figueiredo et al examined the lycopene administration on the diabetic rats. In this study, the authors carried out the trial to examine the synergic effect of dose (insulin) and lycopene.

The authors carried out many experiments using the tissues of the animals in the experiments by measuring some biological parameters. The purpose of the trial is interesting, as authors pointed out in the manuscript, to reduce the dose (insulin) is a quite important point to keep the high-quality life (not fully controlled by insulin).

However, several points are still unclear in the manuscript.

Comment 1

The authors used lycopene in the study. Lycopene is a long-chain hydrocarbon bearing some conjugated and unconjugated double bonds in the molecule. The structure is completely different from that of insulin. The reason for the choice of lycopene in the study is not clear.

Answer:

We are fully in agreement with the reviewer comment. We have included an explanation about the choice of lycopene in the present study.

Please see the new information in the “Introduction” section (page 2, lines 73-78).

Comment 2

Concerning the above point, other carotenoids or xanthine derivatives are also interesting to discuss.

Answer:

In agreement with the reviewer suggestion and following its recommendation, we have included information about other carotenoids.

Please see the new information in the “Introduction” section (page 2, lines 66-68).

Comment 3

It is very important to show the chemical structure of Lycopene for the better understanding of readers.

Answer:

The chemical structure of lycopene was presented in the “Introduction” section (page 2).

Comment 4

In the results section the authors showed the experimental results with brief explanation. The experiments were done six types, however, the explanation (sentences) is quite poor compared with the amount of experimental results

Answer:

We chose to describe the results concisely, in order to highlight the main novelty of the study, that is, the benefits achieved by a combined therapy based on a reduced insulin dose with lycopene. Furthermore, the reviewer made extremely pertinent considerations related to the discussion of the results, which led to substantial changes in the “Discussion” section that much improved the current version of the manuscript.

Comment 5

In the discussion part, the authors did not discuss their experimental results precisely. Namely, the authors discuss the experimental results by quoting several literatures. It is quite important point, however, authors should discuss their original experiments.

Answer:

We completely agree with the reviewer comment. For this reason, as well as considering the comments of the reviewer made individually in the tables and figures, the “Discussion” section was substantially changed, which greatly enriched the current version of the manuscript.

Comment about Table 1

In Table 1, some physical parameters are measured, (body weight, body weight gain, food intake, water intake and urinary volume). It is very clear to find big differences between the negative control (NYOG) and positive control. It is also clear, the administration of 4U insulin (DI4U) improves the values close to NYOG. In the case of DI1U+LYC, the body weight gain is almost same with that of DI4U, however, water intake and urinary volume of DI1U+LYC showed significant differences with those of DI4U. The authors did not discuss these points (including other DYOG, DI1U, and DLYC studies).

Answer:

We completely agree with the reviewer comment. The “Results” section “Combined therapy 1U/day insulin + lycopene prevented loss weight and polyphagia in diabetic rats” addressed the fact that the combined therapy improved the body weight gain and decreased polyphagia in diabetic rats, but this therapy was unable to improve polyuria and polydipsia. In agreement with the reviewer suggestion and following its recommendation, we included a discussion about these findings.

Please see the new information in the “Discussion” section (page 7, lines 580-588).

Comment about Table 2

In Table 2, the authors examined the weights of the tissues of rats. As found in the Table 1, DI4U showed the similar values (gastrocnemius muscle, epididymal adipose tissue, retroperitoneal adipose tissue, and kidneys). DYOG showed significant difference values with those of NYOG. In the case of DLYC, except kidney values, the obtained values are similar to those of DYOG. The values of DI1U and DI1U+LYC showed the values between DI4U and DYOG. Comparing the DI1U and DI1U+LYC values, DI1U+LYC showed the closer value to DI4U than those of DI1U. These experimental results are quite interesting to discuss to investigate the biomarker studies.

Answer:

In agreement with the reviewer suggestion and following its recommendation, we have included information about other beneficial effects of the combined therapy on the weights of adipose and muscle tissues.

Please see the new information in the “Discussion” section (page 7, lines 564-579).

Comment about Figure 2

In Fig. 2, the authors carried out the basal and insulin-stimulated phosphorylation levels of Akt in gastrocnemius muscles of diabetic rats. The sensitivity of DYOG is lower than NYOG, DI4U, DI1U, and DI1U+LYC. The authors got the results based on the densitometric analyses not q-PCR method. In the cases of insulin (-), DI1U showed higher value than that of DI4U. Is it really true? In

this meaning, it is highly recommended to use the q-PCR for the study, as far as the authors discuss the qualitative work, it is meaningful to use densitometry, however, to discuss precisely, it is not adequate to use densitometric values.

Answer:

Unfortunately, the investigation of the changes in the AKT phosphorylation levels is a study of changes at post-translational levels, where an immunoassay is often used. Furthermore, we are currently unable to perform the q-PCR assay in our laboratory. However, we agree with the reviewer that some data must be validated by more than one method.

Comment about Figure 3

In Fig. 3, the amount of triglyceride and cholesterol was increased in the case of DLYC. In Fig. 4, the amount of glycated hemoglobin, fructosamine, and TBARS in blood and plasma is increased in the case of LYC. These results suggest that lycopene does not act as a good antioxidant. While, by the combination with 1U insulin, the corresponding values became lower (almost same as 1U insulin). The explanation for these events must be required.

Answer:

We are fully in agreement with the reviewer comment. We have included a possible explanation for the lack of beneficial effects of lycopene on the biomarkers: glycated hemoglobin, fructosamine, and TBARS.

Please see the new information in the “Discussion” section (page 8, lines 614-620).

Comment about Figure 5

In Fig. 5 (B), the authors drew the urinary albumin amount in Y axis, and indicated significant different marks. The administration of DLYC increase the albumin amount compared with NYOG, D14U, and D1U. The combination of DI1U + DLYC decreased the value lower than those of DI4U, DI1U and NYOG. It is quite interesting observation, however, no explanation for this difference is not shown in the manuscript. The significant difference of DI1U+DLYC have three marks, b, d, e, is it really true? The DI4U value is bigger than DI1U value, so, b, c, d, e (or a, b, c, d, e) might be reasonable.

Answer:

We have included information about the beneficial effects of the combination lycopene + 1U/day insulin on urinary albumin levels, to values below those found in diabetic rats submitted to the standard treatment (4U/day insulin), as well as below those found in non-diabetic rats (NYOG group). Please see the new information in the “Discussion” section (page 9, lines 664-667).

Furthermore, we reviewed the statistics and, in fact, the urinary albumin excretion in DI1U+LYC group is lower than all other groups studied (NYOG, DYOG, DI4U, DI1U, DLYC). Please see the new version of the Figure 5 (page 3).

Comment about Figure 6

In Fig. 6, the authors carried out antioxidant enzymes insulin treated or non-treated rats. In the cases of SOD and CAT, DYOG, DI1U, and DLYC showed the lower values than those of NYOG, while DI4U and DI1U +LYC showed similar values with those of NYOG. The explanation for these events are not shown in the manuscript. In Fig. 5c, the authors indicated the significant different marks of DYOG, DI4U, DI1U, DLYC, and DI1U +LYC as b, is it really true. According to the footnote, mark b showed the significant difference versus DYOG.

Answer:

In agreement with the reviewer suggestion and following its recommendation, we have discussed about the low activities of SOD and CAT found in DYOG, DI1U and DLYC groups. Please see the new information in the “Discussion” section (page 8, lines 624-633).

Furthermore, Figure 6C was corrected regarding the indication of statistical differences between groups, as it had an error. Please see the new version of the Figure 6 (page 4).

Comment about Figure 7

In Fig. 7, the significant increase of AGER-1 / beta-actin was observed in the case of DI1U + LYC (not DLYC in the figure), the clear explanation for this might be necessary.

Answer:

In agreement with the reviewer suggestion and following its recommendation, we have included information about the increase in the levels of AGE-R1 in kidneys of diabetic rats treated with lycopene + 1U/day insulin.

Please see the new information in the “Discussion” section (page 8, lines 651-659; page 9, lines 660-661).

Comment about Figure 8

In Fig. 8 (b), the fluorescent AGEs (kidneys) were measured and similar values were observed in the cases of DYOG, DI4U, DI1U, DLYC, and DI1U+LYC. However, significant differences were found with that of NYOG. The authors carried out the similar experiments using blood sample (Figure 4C). In this case, only DYOG showed the significant difference (high score) to other samples. These tow results showed the different trend, it might be necessary to explain this event.

Answer:

The methodology we use to analyze AGE levels is not sophisticated enough (enzyme-linked immunosorbent assay, high-performance liquid chromatography, liquid chromatography-tandem mass spectrometry) to analyze a specific type of AGE, such as carboxymethyl-lysine, carboxyethyl-lysine methylglyoxal-lysine dimer, N-(5-hydroxy-5-methyl-4-imidazolone-2-yl)-ornithine. The method used in our laboratory detects the fluorescence emitted at 440 nm when AGEs present in the sample are excited at 370 nm. This analytical approach can detect molecules other than just the fluorescent AGEs, since such other molecules can emit at 440 nm when excited at 370 nm, which can be interpreted as analytical interference. Another analytical interference is the fact that other molecules are capable of absorbing the energy emitted by AGEs at a wavelength of 440 nm.

In the case of the kidneys, which are organs responsible for eliminating a large amount of molecules from the body, a plausible explanation for the decrease observed in the levels of fluorescent AGEs in the kidneys of all groups constituted of diabetic animals is the increased presence of molecules that absorb the energy emitted by AGEs at 440 nm. Another interesting explanation is the temporal behavior of glycoxidative stress biomarkers in each organ/tissue during the course of streptozotocin diabetes mellitus in rats, which may be changing over the days.

In the case of the differences observed in the levels of AGEs in plasma samples, the blood receives influences of the AGE levels from different organs/tissues, and we believe that the temporal behavior of glycoxidative stress biomarkers in these organs may be occurring in a direction opposite to what are occurring in the kidneys, that is, higher levels of AGEs may be observed in several other organs (except the kidneys), and including the blood itself, of untreated diabetic rats, with the impacts of treatments being beneficial in terms of reducing glycoxidative stress in tissues, certainly due to the improvements observed in the glycemic control.

Comment 6

In the discussion part, the authors quoted many other researcher's works to strengthen their logic, it is also quite important point. However, at first, the authors should scrutinize the experimental result, and cross-talk the experimental results precisely in the discussion section. Based on these, important literatures must be quoted to deepen the author's logic. The combination of the bioactive compound(s) with bioactive substrate (hormone) is quite important topic to reduce the dose. There are so many choices, so clear logic for the use of bioactive compound(s) should be clarified.

Answer:

The comment of the reviewer was extremely important, which culminated in substantial changes in several parts of the "Introduction" section and especially in the "Discussion" section.

We believe and that the manuscript has been much improved as a whole.

Comment 7

The English usage must be improved. It is very careful to prepare the manuscript before submission. Some grammatical mistakes (not so much) are found in the manuscript. In this meaning, the advice and revision of highly specialized person must be necessary.

Answer:

Extensive revision of the English grammar was performed, in all sections of the manuscript.