Response to the comments of editor and reviewers

February 22, 2015

Dear editor,

Please find enclosed the edited manuscript in Word format (file name: 16404-review.doc).

Title: ameliorative effect of lutein on nonalcoholic fatty liver disease in rats

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Name of Journal: *World Journal of Gastroenterology*

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

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

Reviewer: 03023594

It is a well-designed and thorough study getting promising result. My only question is that there are so many phytochemicals and antioxidants might have health promoting effect, it will be better if the authors could state more circumstantially the reason why they select this particular product.

Response: We want to begin by thanking you for writing that "It is a well-designed and thorough study getting promising result." As the economy has developed, peoples' lifestyles have changed significantly and are characterized by reduced physical activity and a high fat/glucose diet [1]. Lutein is a major carotenoid that is present in dark green leafy vegetables

and various fruits [2, 3]. With a strong blue light filtering ability, lutein is one of the major pigments in the macula lutea on the retina and is found to play a key role in preserving visual performance[4]. Recently, carotenoids have drawn an increasing focus on their role in atherosclerosis (AS). Lidebjer C et al.[5] found that compared to controls, patients with coronary artery disease (CAD) had significantly lower plasma levels of lutein+zeaxanthin and that these low levels were associated with smoking, high body mass index, low high density lipoprotein cholesterol and inflammatory activity; however, plasma levels of hydrocarbon carotenoids (alpha-carotene, beta-carotene, lycopene) in CAD patients had no significant changes. The study indicated that lutein+zeaxanthin may have an important role in the incidence of CAD; however the mechanisms need further study. Moreover, hyperlipidemia, insulin resistance and NAFLD are risk factors of AS. Based on the above understanding, we want to explore the effects of lutein on the risk factors of AS in rats fed a high-fat diet.

References:

1 **Zimmet P**, Alberit KG & Shaw J. Global and societal implications of the diabetes epidemic. *Nature* 2012, 414:782-787[PMID:11742409].

2 **Granado F,** Olmedilla B, Blanco I, Rojas-Hidalgo E**.** Major fruit and vegetable contributors to the main serum carotenoids in the Spanish diet. *Eur J Clin Nutr* 1996;50:246-250[PMID: 8730612].

3 **Sommerberg O,** Keunen JE, Bird AC, van Kuijk FJ. Fruits and vegetables that are source for lutein and zeaxanthin: the macular pigment in human eyes. *Br J Ophthalmol* 1998; 82: 907–910[PMID:9828775].

4. **Kijlstra A,** Tian Y, Kelly ER, Berendschot TT. Lutein: more than just a filter for blue light. *Prog Retin Eye Res* 2012.31:303-315[PMID: 22465791 DOI: 10.1016/j.preteyeres.2012.03.002].

5. **Lidebier C**, Leanderson P, Ernerudh J, Jonasson L. Low plasma levels of oxygenated carotenoids in patients with coronary artery disease. *Nutr Metab Cardiovasc Dis* 2007. 17:448-456[PMID: 17134954].

Reviewer 02944960

The author evaluated the role of lutein supplementation on hepatic fat content and insulin sensitivity measures among high-fat diet fed rats. The results seem quite promising. However the manuscript seems to be severely limited in a number of ways as mentioned below:

(1) Both abstract and manuscript are significantly limited in writing and require significant overhaul in terms of English language. It would be difficult for the English readers to follow the manuscript in its current format. I think the authors should use professional services to improve the language content o f the manuscript.

Response: We highly appreciate the constructive suggestions, which have further improved the quality of our manuscript. We have acquired the services of an English language editing company, American Journal Experts, to assistant us with editing and polishing the language in our manuscript. We have tried our best to improve the manuscript and have included some changes in the manuscript to make it easier to understand for all readers. These changes are not listed here but rather marked in red in the revised manuscript.

(2) There is disparity especially in the methodology section of the abstract and the manuscript. The authors mentioned in the manuscript that the rats were fed high-fat diet for 10 days whereas in the abstract, they mention the duration to be 55 days (page 3, line 59).

Response:

We apologize for the unclear expression. After a careful review of the manuscript, we have clarified, in the methodology section, the duration of high-fat diet feeding and lutein supplementation for rats; we have marked the changes in red in the revised manuscript. Actually, the rats were fed a high-fat diet for 55 days, and they were divided into 4 groups and provided with 0, 12.5, 25 or 50 mg/kg (body weight)/d lutein from day 10 to day 55.

(3) The authors need to clarify in the abstract that the lutein was given from day 10 to day 55 as mentioned in the manuscript section (page 3, line 60).

Response: Thank you for your careful review on our manuscript. As per the reviewer's suggestion, we have now clearly explained the duration of lutein supplementation in the

abstract.

(4) The authors mentioned a number of abbreviations in the abstract without explaining them first. Please explain the abbreviations first and then they can be used as such later in the abstract and manuscript sections.

Response: Thank you very much for the good suggestion. We have now explained the abbreviations in the abstract.

(5) The authors need to clarify further how the 4 groups were made based on serum cholesterol level (page 7, line 180).

Response:

After supplementation with a high-fat diet for 10 days, the level of TC increased significantly. Then, the rats fed the high-fat diet were divided randomly into 4 groups according to the levels of their TC. As shown in figure 1, within all high-fat diet groups, serum TC was significantly higher (P<0.01) than that in the normal diet group, and there was no significant difference in serum TC in the 4 groups.

(6) The analysis plan needs to detail further in the statistical analysis portion. The authors should name statistical tests that were used to compare various groups.

Response:

Thank you for your professional advice. We have now listed the statistical tests that were used to compare various groups in the statistical analysis portion.

(7) The baseline characteristics of the rats in term of weight and cholesterol levels prior to start of high-fat diet should be mentioned. If the rats were obese to begin with, high-fat diet may worsen already existing NAFLD in these rats.

Response:

Thank you for your insight that indicated that the rats might be obese at the beginning of the study. In our study, the rats were fed a normal diet for a 9-day acclimation period before the beginning of our research. After 9 days of acclimation, the weights of rats in the two

groups were 201 g and 199 g. Then, the rats in the two groups were fed a normal diet or a high-fat diet for 10 days, and the weights increased to 254 g and 262 g, respectively. However, we did not measure serum TC after the acclimation period. Based on our experience, there is no significant difference in the blood lipid levels of animals from the same group. Nevertheless, from the perspective of rigorous scientific research, serum TC should be determined. We will definitely keep in mind for our future research.

Table 2 Body weights of rats at different time-point

Group	weight(g)	
	9 days	19 days
ND	201±7.05	254±8.90
HFD	199±7.59	262±15.09

Values are the mean ± SD. After 9 days of acclimation, the rats were randomly divided into the ND (n=8) and HFD (n=32) groups. Then, the rats in the HFD group were fed a high-fat diet for 10 days.

(8) Is it possible to measure the size of reduction of liver fat content (figure 5 in terms of percentage reduction) in rats receiving Lutein supplementation?

Response:

Thank you for the insightful suggestion on analysis methods. Based on the suggestion, we referred to some studies and found that the method was used by Chalkiadaki A et al.[1] on analysing the size of fat cells in adipose tissue, however it seems to be unsuitable for liver tissue. Based on other studies and our experience[2,3], the quantification of hepatic steatosis in animal models has often used Oil Red O staining to observe lipid droplets (area fraction), this method has been demonstrated as a good control for triglyceride stores. We will investigate this in our future research.

References:

1 **Chalkiadaki A**, Guarente L. High-fat diet triggers inflammation-induced cleavage of SIRT1 in adipose tissue to promote metabolic dysfunction. *Cell Metab*. 2012. 16:180-188[PMID: 22883230 DOI: 10.1016/j.cmet.2012.07.003].

2 Guo R, Xu X, Babcock SA, Zhang Y, Ren J. Aldehyde dedydrogenase-2 plays a beneficial

role in ameliorating chronic alcohol-induced hepatic steatosis and inflammation through regulation of autophagy. *J Hepatol*. 2015.62: 647-656[PMID: 25457208 DOI: 10.1016/j.jhep.2014.10.009].

1 **Levene AP**, Kudo H, Thursz MR, Anstee QM, Goldin RD. Is oil red-O staining and digital image analysis the gold standard for quantifying steatosis in the liver? *Hepatology*. 2010. 51: 1859[PMID: 20432267 DOI: 10.1002/hep.23551]

(9) The results are not consistent for increasing concentrations of Lutein on serum and liver cholesterol parameters. The authors should explain the disparities in these results? Lutein 50 mg seems to be working on liver total cholesterol, but has not effect on liver triglyceride or any of the serum cholesterol parameters (table 2).

Response:

Thank you for your insightful review. This is a worthwhile thought that has also been troubling us. After first noticing this finding, we measured these indexes again, and the results were consistent with those measured before. By referring to other studies, we found that this result might be due to the hepatic adaptive adjustment of lipid metabolism. The levels of total cholesterol in serum and liver might indicate different stages of lipid metabolism [1, 2, 3]. In addition, the doses of lutein supplementation might not be the best doses. For the dosage design, we referred to other researchers and expected to obtain a good dose-dependent effect of lutein supplementation on NAFLD, but our results didn't show the dose-response relationship well. This result prompted us to give more consideration to the dose design of lutein supplementation in our future research.

References:

1 **Zhukova NV**, Novqorodtseva TP, Denisenko YK. Effect of the prolonged high-fat diet on the fatty acid metabolism in rat blood and liver. *Lipids Health Dis*. 2014. 13: 49 [PMID: 24628762. DOI: 10.1186/1476-511X-13-49].

2 **Okada Y**, Yamaquchi K, Nakajima T, Nishikawa T, Jo M, Mitsumoto Y, Kimura H, Nishimura T, Tochiki N, Yasui K, Minami M, Kaqawa K, Okanoue T, Itoh Y. Rosuvastain ameliorates high-fat and high-cholesterol diet-induced nonalcoholic steatohepatitis in rats. *Liver Int*. 2013.33: 301-311[PMID: 23295058. DOI: 10.1111/liv.12033].

3 **Biddinger SB**, Almind K, Miyazaki M, Kokkotou E, Ntambi JM, Kahn CR. Effects of diet and genetic background on sterol regulatory element-binding protein-1c, stearoyl-CoA desaturase 1, and the development of the metabolic syndrome. *Diabetes*. 2005.54: 1314-1323[PMID: 15855315].

(10) Figure 2. The total cholesterol and triglyceride levels of HFD rats are statistically different compared to the ND group as per table 1. What does b indicate in figure 2?

Response: We apologize for forgetting to explaining b in Figure 2. It indicates $P<0.01$ compared to the ND group, and we have marked this change in red in the revised manuscript.

(11) It seems that HOMA-IR, HOMA B, IRS2, P13K, GLUT2, PPAR-α and SIRT1 measurements were performed in only small number of rats (figure 6, 7 and 8). How these rats were selected for the analysis? This will be a major limitation of this study.

Response:

Thank you for your profound insight and for pointing out a major issue in the manuscript. In our study, the HOMA-IR and HOMA-β of all rats were calculated according to their formulas. Then, we chose 4 rats for RT-PCR analysis and 3 rats for western blot analysis randomly based on other studies and our experience[1, 2, 3]. However, some studies chose more rats for RT-PCR and western blot analyses[4, 5]. According to our results and the recommendations of the reviewer, we will enlarge the sample size in our future research.

Reference:

1 **Jwa H**, Choi Y, Park UH, Um SJ, Yoon SK, Park T. Piperine, an LXRα antagonist, protects against hepatic steatosis and improves insulin signaling in mice fed a high-fat diet. *BiochemPharmacol*. 2012. 84:1501-1510 [PMID: 23000915. DOI: 10.1016/j.bcp.2012.09.009].

2 **Selvarai RK**, Shanmuqasundaram R, Klasing KC. Effects of dietary lutein and PUFA on PPAR and RXR isomer expression in chickens during an inflammatory response. *Comp Biochem Physiol A Mol Integr Physiol*. 2010. 157: 198-203 [PMID: 20601055. DOI: 10.1016/j.cbpa.2010.06.172].

3 **Budick-Harmelin N**, Anavi S, Madar Z, Tirosh O. Fatty acids-stress attenuates gluconeogenesis induction and glucose production in primary hepatocytes. *Lipids Health Dis*. 2012. 11:66[PMID: 22676303. DOI: 10.1186/1476-511X-11-66]

4 **Chen S**, Li J, Zhang Z, Li W, Sun Y, Zhang Q, Feng X, Zhu W. Effect of resveratrol on the amelioration of insulin resistance in KKAy mice. *Can J Physiol Pharmacol*. 2012. 90: 237-242 [PMID: 22309033. DOI: 10.1139/y11-123].

5 **Wang HN**, Wang YR, Liu GQ, Liu Z, Wu PX, Wei XL, Hong TP. Inhibition of hepatic interleukin-18 produciton by rosiglitazone in a rat model of nonalcoholic fatty liver disease. *World J Gastroenterol*. 2008. 14: 7240-7246[PMID: 19084941].

(12) It will be better to have actual values for comparison rather than just mentioning p values in the result section.

Response: We fully agree with your recommendation, and we have now used some actual values for comparison instead of just mentioning p values in the results section.

(13) Page 11, line 289: $p<0.01$ is for 25 mg/kg instead of 12.5 mg/kg as per figure 6.

Response: Thank you for your careful review. We have corrected the mistake on page 11, line 289 and marked the change in the revised manuscript.

(14) Figure 7: The authors mentioned that mRNA expression was upregulated significantly in P13K and GLUT2 (fig 7B and 7C). The protein expression was also regulated (fig 7D, 7E and 7F) in the lutein fed rats, were they were statistically significant ($p<0.05$)?

Response: Thank you very much for pointing out the shortcomings of our research. In our study, the result from the mRNA expression analysis was statistically significant. While the result was not statistically significant, lutein supplementation had a potential effect on protein expression. The non-significant result might be due to the small number of samples (n=3) or due to the large standard deviation. Therefore, we will increase the number of samples to obtain more precise results in our future research.

(15) The authors should mention potential limitations of the study.

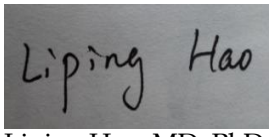
Response:

Thank you again for your careful and insightful suggestions. We have mentioned potential limitations of the study and have marked them in red in our revised manuscript. In our study, the number of rats we chose for RT-PCR and western blot analyses was rather limited, we will increase the sample size in future research. In addition, the doses of lutein supplementation need more consideration in our future studies.

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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