ANSWERING REVIEWERS

November 12, 2013

Dear Jin-Lei Wang,

Please find enclosed the edited manuscript in Word format (file name: 5949-review.doc).

Title: Exenatide improves hepatic steatosis by enhancing lipid use in adipose tissue in nondiabetic rats (Previous title: Exenatide improves hepatic steatosis by enhancing lipid use in adipose tissue)

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Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 5949

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

1 Format has been updated.

2 Revisions have been made according to the suggestions of the reviewers.

(1)Reviewer No. 00038192

1. The abbreviation GLP-1 has to be explained before used the first time. Exenatide hast to be shortly introduced.

→As the reviewer suggested, we defined GLP-1 as “glucagon like peptide-1” in the Core tip. In the Introduction section, we describe it on page 4, lines 9 to 12. We inserted a description of exenatide in the Introduction section, page 4, lines 12 to 15.

2. Superoxide dismutase, please specify which of the 3 proteins.

→We investigated the expression levels of SOD2. We corrected the descriptions in the text and Fig. 5B.

3. Abstract: “were subdivided into two groups and injected with either exenatide or saline every day for 12 weeks. The control group received saline alone “are there 2 control groups?

→We used a single control group. To make the groups clear, we renamed the groups as follows: control group, fed normal chow diet during all of the periods and injected with saline every day after 4 weeks from the beginning of the experiment; HFD-Ex (-) group, fed high-fat diet during all of the periods and injected with saline after 4 weeks from the beginning of the experiment; and HFD-Ex (+) group, fed high-fat diet during all of the periods and injected with exenatide after 4 weeks from the beginning of the experiment. We corrected the description in the Abstract (page 3, lines 5 to 9), Materials and Methods section (page 5, lines 15 to 25), Figures, and Figure legends.

4. Results “Enhanced lipid oxidation results in the accumulation of intracellular reactive oxygen

species (ROS), which induces the cellular response to eliminate this harmful by-product." Please give references

→As the reviewer suggested, we added the appropriate references on page 18, line 26 to page 19, line 7.

(2)Reviewer No.02461842

1. The authors should challenge the mice also with a high carbohydrates diet and see whether the effect on the GLP-1 is the same as for the high fat diet.

→We agree with the reviewer's suggestion. It is very interesting whether the effects of GLP-1 on reducing hepatic lipid contents would be influenced by the pathogenesis of NAFLD. However, we speculate that the NAFLD model induced by a high carbohydrate diet would easily become diabetic, and correction of blood glucose by GLP-1 would modify the hepatic steatosis. Because we intended to investigate the action of GLP-1 in a nondiabetic status, we have considered that this study should be performed using a nondiabetic high-fat diet-induced NAFLD model. When the detailed mechanism of GLP-1 in this model is fully understood, comparison of the effects of GLP-1 among the models with different pathogenesis should be investigated.

2. The authors should also measure the Short chain fatty acids (butyrate, acetoacetate and propionate) and check whether these might be related to GLP-1 expression.

→We agree with the reviewer's comment. Because short chain fatty acids are reported to stimulate GLP-1 secretion from intestinal L cells, elevation of short chin fatty acids induced by enhanced lipolysis may change the endogenous levels of GLP-1. However, animals in this study were treated with relatively large amounts of exenatide, an exogenous GLP-1, and changes in endogenous GLP-1 levels might be negligible. In addition, unfortunately, we do not have the opportunity to use equipment, e.g., HPLC or gas chromatography, to evaluate short chain fatty acids. Therefore, we regret to say that the levels of short chain fatty acids are currently unable to be analyzed in this study.

3. There are several typos.

→We identified the typographical errors and corrected them.

(3)Reviewer No. 01800329

1. The details of assessment of histological changes in the animals are not given – e. g. quantification of the changes in the liver, and the details of areas chosen for analysis in the adipose tissue and the number of adipocytes assessed etc. These need to be provided.

→As suggested, we evaluated the number of lipid droplets in liver sections and reported them in Fig. 3C, and they are described in the Results section, page 8, lines 11 to 14. With regard to the adipose tissue, we chose epididymal adipose tissues for analyses of the diameters of adipocytes and inserted the description in the Materials and Methods section, page 6, line 7. We evaluated the diameters of 100 adipocytes in each animal (n=5/group). We described this in the Materials and Methods section, page 6, lines 11 to 13.

2. How was the energy intake measured in the animals between Weeks 6 and 12 of the feeding protocol? Was the energy intake significantly different in the 3 groups?

→We maintained two rats in a cage (four cages in each group) and measured the daily food consumption in each cage. Therefore, we could evaluate the average level of energy intake in each group but could not analyze the differences statistically. We described this in the Materials and Methods section, page 5, lines 23 to 25.

3. What statistic was used to compare 2 groups e. g., as with Oxygen consumption and RER?
→We statistically compared 2 groups using Kruskal-Wallis test. We inserted the description in Materials and methods section, in page 7, lines 15 to 17.

4. In the text and figures, the saline treated HFD group is referred to as HFD whereas the exenatide group is referred to as exenatide group while this group too was on HFD. Similarly rats on control diet are referred to as the 'control group', while the rats on HFD injected with saline also form a control group. This creates confusion – and is better corrected.
→As pointed out, the present names of groups seem to be confusing. Therefore, we corrected the group names as follows: control group, fed normal chow diet during all of the periods and injected with saline every day after 4 weeks from the beginning of experiment; HFD-Ex (-) group, fed high-fat diet during all of the periods and injected with saline after 4 weeks from the beginning of experiment; and HFD-Ex (+) group, fed high-fat diet during all of the periods and injected with exenatide after 4 weeks from the beginning of the experiment. We corrected the description in the Abstract (page 3, lines 5 to 9), Materials and Methods section (page 5, lines 15 to 25), Figures, and Figure legends.

5. The authors hypothesized that exenatide effected its changes through altered lipid metabolic activities, including triglyceride hydrolysis and lipid oxidation. Could there be an alternative explanation – for e. g. restlessness or hyperactive behavior in the animals?
→We agree with the reviewer's comment. If GLP-1 induces hyperactive behavior in treated animals, enhanced energy consumption would result in reduced hepatic lipid content. However, hyperactive behavior by GLP-1 has not been reported. Although GLP-1 improved cognitive function in obese mice (International Journal of Obesity 34, 1341-1344, 2010) and Alzheimer's disease (Journal of Neuroscience 31, 6587-6594, 2011), GLP-1 did not alter locomotor activity in obese mice (Peptides 35, 1-8, 2012) and rats (Neuropeptides 44, 285-91, 2010). In our experiments, we did not evaluate the locomotive activities, but specific behavioral differences were not observed among the groups. We considered the possibility that GLP-1 modified the activity of animal's behavior may still remain, but the observations to support this hypothesis seem to be insufficient. To avoid confusion, we did not include this hypothesis in this manuscript.

6. On treatment with exenatide the animals exhibited decreased food intake, weight loss, increased oxygen consumption, decreased respiratory exchange ratio, less liver fat and increased lipid oxidation by adipocyte? The authors do not tie up these findings to provide a satisfactory explanation for the effects of the drug in the discussion. This needs to be done.
→We agree with the reviewer's comment. The enhanced lipolysis in white adipose tissue might not be solely sufficient to increase systemic energy consumption. Recently, Lockie et al. reported that intracerebroventricular injection of GLP-1 induced thermogenesis in brown adipose tissue, accompanied with increased activity of innervated sympathetic fibers (Diabetes 61, 2753-62, 2012). Indeed, enhanced thermogenesis seems to increase energy consumption, resulting in body weight reduction. In this study, we tried to evaluate the mRNA expression levels of thermogenesis-related genes in brown adipose tissue; however, handling difficulties of this tissue did not allow us to obtain accurate measurements. Although we are unable to show our own data, we describe the possibility of the involvement of brown adipose tissue in the Discussion section, page 13, lines 12 to 20.

7. HFD did not increase the weight in the saline treated group. And yet the animals on HFD

developed hepatic steatosis. Exenatide prevented the steatosis. What is the significance of this model to human NAFLD?

→While most of the investigations for NAFLD were performed in high-fat diet (HFD)-induced obese animal models, we often find reports describing the presence of hepatic steatosis in HFD models without obesity (Hypertension 62, 324-330, 2013, Journal of Oleo Science 62, 57-64, 2013). Specifically, HFD with a high cholesterol diet is known to induce NAFLD without obesity (Hepatology 46, 1392-403, 2007, Journal of Gastroenterology 41, 971-980, 2006). In humans, significant numbers of lean subjects were diagnosed as NAFLD, even if the prevalence was less than in obese subjects (Archives of Internal Medicine 164, 2169-2175, 2004). A nutritional study showed that dietary cholesterol levels were higher in nonobese NAFLD subjects than in obese NAFLD subjects (Scandinavian Journal of Gastroenterology 44 471-477, 2009). From these observations, we may postulate our model as a nonobese NAFLD model mimicking lean NAFLD patients and advocate the use of GLP-1 in these patients. However, some of these lean NAFLD patients are supposed to be influenced by genetic background, e.g., polymorphisms in MTP or PNPLA3. Hence, the pathogenesis of nonobese NAFLD might be more complicated than in animal models. Therefore, we consider that describing the use of GLP-1 in nonobese NAFLD in this manuscript would be premature.

8. Modifying the title to include 'non-diabetic rats' would put the whole paper in perspective.

→As suggested, we have corrected the title as follows "Exenatide improves hepatic steatosis by enhancing lipid use in adipose tissue in nondiabetic rats".

(4)Reviewer No. 00058403

1. ***They were not put in abstract values and p-values found in the results, and the main aim is not according to the study.***

→We have added the p-values to the Abstract section, and values and p-values to the Results section. Moreover, to describe the aim more precisely according to this study, we changed the sentence "To investigate the role of skeletal muscle and/or adipose tissue…" into "To investigate the metabolic changes in skeletal muscle and/or adipose tissue…" in the Abstract section.

2. In the introduction, the last paragraph is confusing. The authors describe the hypothesis and results of this paper, without making clear the aim of the study.

→We agree with the reviewer's suggestion. We modified the last paragraph in the Introduction section, focusing on the metabolic changes in adipose tissue by GLP-1, page 4, line 28 to page 5, line 8.

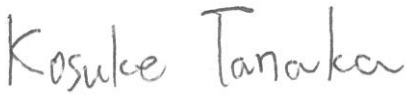
3. The results were also not identified the values corresponding to the analyses made in the study (data not shown in figures).

→We have added the values and p-values to the Results section corresponding to the analyses.

4. References and typesetting were corrected.

Thank you again for reviewing our manuscript for publication in the *World Journal of Gastroenterology.*

Sincerely yours,



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