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Dear Editor Veronika Somoza,

We thank you very much for giving us the opportunity to revise our manuscript and really appreciate the constructive comments and suggestions by you and the reviewers for our manuscript (Article reference: *jf-2021-00726h*) entitled "**Hydrophobic BCAA peptides mediate the hypoglycemic effect via activating PI3K/Akt signaling**" which now entitled "**The hypoglycemic effect of hydrophobic BCAA peptides is associated with altered PI3K/Akt protein expression**". We have carefully revised the manuscript to address the reviewers' comments.

We believe now we have completely addressed yours and the reviewers' comments. Those comments are all valuable and very helpful for revising and improving our manuscript, as well as the important guiding significance to our researches. Revisions of manuscript are highlighted in red for your reference. Below please check for our response to yours and the reviewers' comments point-by-point, which are also reflected in our revised manuscript. The comments are in black italicized font and our responses are in blue font.

Please kindly let us know if you have any questions and we look forward to your positive consideration!

With best regards,

Yours sincerely,
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Point-by-Point Response to Editor's and Reviewers' Comments

(*Bold black italic: Reviewer's remarks*; Blue type: Our response)

List of Responses

Dear Editors and Reviewers:

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "**The hypoglycemic effect of hydrophobic BCAA peptides is associated with altered PI3K/Akt protein expression**" Manuscript ID: *jf-2021-00726h*. Those comments are all valuable and very helpful for revising and improving our paper. We have studied comments carefully and have made correction which we hope meet with approval. Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the reviewer's comments are as flowing:

Editor:

Manuscript Format: Manuscripts are to be double-spaced and line-numbered. A separate summary or conclusion section is not to be used; any concluding statements are to be incorporated under Results and Discussion. Please make sure to remove any track changes, highlights, comments or font colors in your manuscript file.

Response: Thank you very much for reminding. We have made the manuscript double-spaced and line-numbered. A separate summary or conclusion section isn't used; all concluding statements are incorporated under Results and Discussion. We have made sure to remove any track changes, highlights, comments or font colors in our manuscript file.

Table of Contents Graphic: The TOC graphic must be included. Authors of research articles, perspectives, and reviews are required to include a suitable graphic for publication in the table of contents (TOC) in the Web edition of the Journal. This graphic should capture the reader's attention and, in conjunction with the manuscript's title, give the reader a quick visual impression of the type of chemistry described. The TOC graphic may be up to 3.33 in. (8.47 cm) wide and 1.88 in. (4.76 cm) tall. (See detailed instructions at the Paragon Plus Web site.) Text should be

limited to labels for compounds, reaction arrows, and figures. The use of color to enhance the scientific value is encouraged. The TOC graphic should be inserted on a separate page at the end of the manuscript file.

Response: Thank you very much for reminding. The TOC graphic for the manuscript has been included in accordance with requirements and submitted with the manuscript.

Reviewer #1:

Comments:

The revised report by Zhu et al. now entitled "Hydrophobic BCAA peptides mediate the hypoglycemic effect via activating PI3K/Akt signaling" under consideration for publication in the Journal of Agriculture and Food Chemistry examined the effect of various BCAA-containing peptides isolated from seabuckthorn on indicators of insulin resistance in db/db mice. In general, the revised report has addressed many of the comments from this reviewer, however the authors need to still consider the following points.

Response: We greatly appreciate the reviewer's positive comments.

Major Comments

The authors describe the involvement of the PI3K/Akt/Glut4 pathway as a "likely" potential mechanism, but given limitations of the study, in this reviewer's mind it cannot be concluded what the likely mechanism is. As pointed out by reviewer #2, perhaps the peptides are functioning like a TZD which would also explain the concurrent weight gain with improved glucose homeostasis (this was not explored and should be included in the limitations section).

Response: Thanks for your constructive suggestions, which we totally agree. We have added a deeper rationale in the discussion section on the concurrent weight gain with improved glucose homeostasis. BCAA supplementation can increase PPAR-γ expression in white adipose tissue of db/db[30]. Therefore, BCAA peptides can increase

the body weight by efficient lipid storage in adipocytes. This point to the possibility that BCAA peptides reduce the accumulation of lipids in muscles by promoting more effective storage of lipids in adipocytes, and restore the effect of IRS-1 and improve insulin sensitivity by reversing the lipid content in muscles (Line 327-333).

(30) Terakura, D.; Shimizu, M.; Iwasa, J.; Baba, A.; Kochi, T.; Ohno, T.; Kubota, M.; Shirakami, Y.; Shiraki, M.; Takai, K.; Tsurumi. H.; Tanaka, T.; Moriwaki, H. Preventive effects of branched-chain amino acids supplementation on the spontaneous development of hepatic preneoplastic lesions in C57BL/KsJ-db/db obese mice. *Carcinogenesis*. **2012**, *33*, 2499-2506.

For this reason, the authors should state that improvements were associated with changes in protein expression in the PI3K/Akt/Glut4 pathway (because without GLUT4 protein expression or measurement of insulin response via measurement of pAKT from experiments as described by reviewer #2, it is also difficult to say that altered insulin sensitivity or that "activation" of the Akt pathway occurred as described in the title and in lines 338-340). This should be adjusted within the title, abstract, and in lines 338-340 and 342-343. Given that "activation" was not measured and that the dependency of resolved blood glucose/HOMA-IR on the PI3K/Akt/Glut4 pathway was not assessed, the most appropriate description in this reviewer's mind is that: "The hypoglycemic effect of hydrophobic BCAA peptides is associated with altered PI3K/Akt protein expression".

Response: Thanks for your constructive questions and suggestions, which we totally agree. We have revised the entitled "Hydrophobic BCAA peptides mediate the hypoglycemic effect via activating PI3K/Akt signaling" by "The hypoglycemic effect of hydrophobic BCAA peptides is associated with altered PI3K/Akt protein expression". We have adjusted abstract (Line 26) and discussion (Line 354).

Lines 207-211 The authors describe ANOVA for the analysis of choice for these data, but do not describe the correction for multiple comparisons (such as Bonferroni's or Tukeys). This correction should be included and described both in the statistical methods and within each figure/table legend.

Response: Thanks for providing constructive suggestions. We have described the

correction for multiple comparisons (Line 212-213). This correction also have included and described both in the statistical methods and within each figure/table legend.

The authors have also resolved many of the typographical errors from the original manuscript, and the paper is much clearer. However, the authors should also correct the following typographical errors.

Response: We greatly appreciate the reviewer's positive comments.

Line 62- A "sink" doesn't seem like the best analogy for muscle as a cite for glucose metabolism/storage. Perhaps reword by stating that "skeletal muscle is a primary site for glucose metabolism and storage, and is also a tissue that is highly susceptible to insulin resistance.".

Response: Thanks for the reviewer's constructive suggestions. The previous statement of "Muscle, the primary sink for glucose after a glucose load, is the predominant site of insulin resistance." was changed to "Skeletal muscle is a primary site for glucose metabolism and storage, and is also a tissue that is highly susceptible to insulin resistance."(Line 62-62).

Line 237 should read "Notably, after BCAA peptide administration"

Response: Thanks for the reviewer's constructive suggestion. The sentence of "Notably, after BCAA peptides administration" was replaced with "Notably, after BCAA peptide administration" (Line 240).

Line 264- "peptides" should be replaced with "peptide"

Response: Thanks for the reviewer's constructive suggestion. The word "peptides" was replaced with "peptide" (Line 267).

Line 273 which reads "However, the difference between the BCAAM and BCAAL groups was insignificant ($p > 0.05$)." is unclear as written (and possibly inaccurate).

The previous statement is referring to differences in both insulin and HOMA-IR, and there is a difference between BCAAM and BCAAL in HOMA-IR. There are also differences between both group and the DC in HOMA-IR. Please reword this sentence for clarity.

Response: Thanks for the reviewer's helpful suggestion. This sentence has been reworded for clarity (Line 276-280).

Line 338- the word "the" should be removed.

Response: Thanks for the reviewer's helpful suggestion. The word "the" has been removed.

Reviewer # 2:

Comments:

Thank you for responding to my previous comments. There are still some outstanding points that need to be addressed.

Response: Once again, we greatly appreciate the reviewer's positive comments and constructive suggestion.

1. Significant figures in abstract. This should be revised to 78.8 ± 1.4.

Response: Thanks for the reviewer's constructive suggestion. We have revised 78.78 ± 1.37 to 78.8 ± 1.4. (Line 15-16).

2. Definition of animal groups. This is still confusing. The DC and NC groups are defined identically in the methods (lines 133 and 138). Adding to this confusion is the statement in line 132 that all the groups were db/db mice. In fact, the normal group comprised hemizygous mice

Response: Thanks for the reviewer's helpful comments. We have re-described the definition of animal groups (Line 133, 139).

3. The discussion on the mechanism of action of BCAA is still superficial and is confusing.

Response: Thanks for the reviewer's helpful comments. Given the limitations of our

research, we have described the participation of the PI3K/Akt/Glut4 pathway as a "possible" underlying mechanism.

Now that it is established that pioglitazone was used in the study (i.e. not metformin) there should be some rationale as to why this treatment was included? Currently, there is no context at all.

Response: Thanks for the reviewer's helpful comments. The positive control group of our study is pioglitazone, and metformin was wrongly written in the original manuscript, which has been corrected. In addition, our results have proven that pioglitazone strongly improved the downstream PI3K/AKT signaling pathway, which was also validated by several studies[1-2]. Therefore, this method should be included.

[1] Nathan, T. I.; Robert, L.; David, S.; Junghwan, O.; Robert, J. H.; Peter, W.; Lynn, k.; Garth, P. Peroxisome proliferator-activated receptor ; γ agonist pioglitazone prevents the hyperglycemia caused by phosphatidylinositol 3-kinase pathway inhibition by PX-866 without affecting antitumor activity. *Mol. Cancer Ther*. **2009**, *8*, 94–100.

[2] Xing, B.; Xin, T.; Hunter, R. L.; Bing, G. Pioglitazone inhibition of lipopolysaccharide-induced nitric oxide synthase is associated with altered activity of p38 MAP kinase and PI3K/Akt. *Journal of Neuroinflammation.* **2008**, *5*, 1-11.

The discussion also pointedly implies that the effects of BCAAs on skeletal muscle are direct yet the reference I provided in my previous response (I am not an author on this paper) suggests that BCAAs also increase PPAR-gamma expression in white adipose tissue of db/db [see Terakura et al. (2012). This points to the possibility that the actions of BCAAs on skeletal muscle are indirect if lipid accumulation in muscle is attenuated by more efficient lipid storage in adipocytes . As long chain CoA species inactivate IRS-1, reversing lipid content in muscle would restore IRS-1 action and explain the current findings. Overall, a deeper rationale is needed here.

Response: Thanks for the reviewer's constructive suggestions, which we totally agree. We have added a deeper rationale in the discussion section. BCAA supplementation can increase PPAR-γ expression in white adipose tissue of db/db[30]. So, BCAA peptides can increase the boy weight by efficient lipid storage in adipocytes. This point to the possibility that BCAA peptides reduce the accumulation of lipids in muscles by promoting more effective storage of lipids in adipocytes, and restore the effect of IRS-1 and improve insulin sensitivity by reversing the lipid

content in muscles (Line 327-333).

(30) Terakura, D.; Shimizu, M.; Iwasa, J.; Baba, A.; Kochi, T.; Ohno, T.; Kubota, M.; Shirakami, Y.; Shiraki, M.; Takai, K.; Tsurumi. H.; Tanaka, T.; Moriwaki, H. Preventive effects of branched-chain amino acids supplementation on the spontaneous development of hepatic preneoplastic lesions in C57BL/KsJ-db/db obese mice. *Carcinogenesis*. **2012**, *33*, 2499-2506.

The authors cite reference 32 which suggests direct actions of pioglitazone on L6 muscle cells so perhaps both direct and indirect mechanisms may be at play?

Response: Thanks for your constructive questions. We cite reference 32 (now 33) which suggests direct and indirect actions of pioglitazone on L6 muscle cells. For instance, as a synthetic ligand for PPARγ and increasing PI3K/Akt. So, the role of pioglitazone perhaps both direct and indirect mechanisms may be at play.

4. Following from the above, the lack of direct studies of BCAAs on muscle should be included in the limitation section of the discussion.

Response: Thanks for your constructive questions and suggestions, which we totally agree. We have included the lack of direct studies of BCAAs on muscle in the limitation section of the discussion (Line 363).

5. The manuscript graphic is misleading as it also implies that the actions of BCAAs on muscle are direct. Perhaps the left hand side of the graphic could have the BCAA and solid arrows removed. Instead arrows could indicate increased action of PI3K and IRS-1? The graphic would then show that BCAA administration to mice (right side of graphic) leads to activation of PI3K/Akt signaling without explicitly implying a direct action.

Response: Thanks for your constructive suggestions, which we totally agree. The manuscript graphic has been revised according to the reviewer's suggestions.

6. In my previous comments I referred to confusion around the letters for significance in the Figures. Aspects of this are still confusing. For example, for Figure 4, the DC group all have "b". By the definition that different letters indicate significant differences, this would mean that the three column bars for the DC group are not significantly different. Yet, visual inspection suggests they very clearly are?

Response: Thanks for your constructive questions and picking up the mistakes, which

we totally agree. We have reworded “Bars with different letters indicate a significant difference ($p < 0.05$).” by “Different letters within the same fill color bar indicate a significant difference ($p < 0.05$).” (Line 521 and 530).

From the Editor's point of view, the following point shave to be addressed:

1. Please add the effective dose of BCAA peptides in the TOC graphic.

Response: Thanks for your constructive suggestions, which we totally agree. We have added the effective dose of BCAA peptides in the TOC graphic.

2. Please include an effect size of the BCAA peptides in the abstract, e.g. for the effect on muscle glycogen contents.

Response: Thanks for your constructive suggestions, which we totally agree. We have included an effect size of the BCAA peptides in the abstract, e.g. for the effect on muscle glycogen contents (Line 19, Line 21-22).

3. Statistics: Please provide the post-hoc test applied following ANOVA analysis.

Response: Thanks for your constructive suggestions, which we totally agree. We have provided the post-hoc test applied following ANOVA analysis (Line 212-213).

4. Results: Please provide the purity of the BCAA peptides (page 12).

Response: Thanks for your constructive suggestions, which we totally agree. We have provided the purity of the BCAA peptides (page 12) (Line 226; Line 228).