Dear Editor and reviewers,

Thank you for considering our manuscript for publication in Cells. We are very pleased to have been given the opportunity to revise our manuscript, “Effect of Dietary Silk Peptide on Obesity, Hyperglycemia, and Skeletal Muscle Differentiation in High-fat Diet-Fed Mice”. We have addressed the reviewer’s comments point-by-point and made the necessary changes to the manuscript.

In major revision, we have addressed the reviewer’s comment about method of grip strength test, experiment to investigate the body temperature, and explanation of muscle data implying that SP has a potential to treat sarcopenia using 6-week-old mice and 12-month-old mice. Therefore, we revised regarding data and added supplementary data and related description in result and discussion section, and highlighted the changes with track changes in the manuscript.

We hope that the manuscript is acceptable for publication in Cells. We appreciate to consider this paper for publication in Cells, and declare that authors of this work have no conflict of interests.

Sincerely yours,

Boo-Yong Lee, Ph.D.

Revision of Cells-702946

Reviewer2

1-Your mice on HF diet had a weight gain of 25g in 6 weeks which is considerable. How many animals per cage? At what temperature the animals housed?

Answer) We appreciate your suggestion. As reviewer’s comment, we revised as “The mice were initially housed for 1 week under a 12 h light/dark cycle condition in 20-24 °C temperature and 44-52% humidity to permit adaptation. Four male mice were kept in each cage used in the study. After adaptation period, the mice …” in 2.2 Animals and experimental design on page 3.

2-In what nutritional state the mice were euthanized? In fasting or feeding condition? This is very important for the subsequent analyses of insulin signaling pathway performed on VAT and on skeletal muscle.

Answer) As reviewer’s comment, we corrected in as “At the end of the experimental period, the mice were fasted for 12 h and euthanized using gradual-fill method of carbon dioxide euthanasia, and their tissues were collected for analysis.” on page 3

3-The authors found SP administration induced a resistance to obesity on HF diet but they indicated that the inhibition of body mass gain in SP-treated mice was not due to lower food intake. In this case, the weight loss is due either to a sharp increase in basal metabolism or a drop in intestinal absorption or a combination of both.

In order to answer this central question, metabolic analyses of these mice are necessary, for example using the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments). Have you studied body temperature in your mice?

Answer) Thank you for your suggestion. We have been tried to investigate the lipid metabolism as your
advice. However, we could not analyze the metabolic change using the CLAMS due to financial problem. Thereby, we measured the body temperature of the mice to investigate the effect of SP on energy expenditure, instead of CLAMS. We added this data as Figure 1I, and explained the result:

“Lastly, to investigate the effect of SP on energy expenditure, rectal temperature of the mice was measured at the end of the administration period. As a result, SP significantly increased body temperature to 38.0 ± 0.4 and 38.0 ± 0.3 °C at 50 and 200 of SP treated group, respectively.” -in Result 3.1.

"Moreover, SP administration increased the rectal temperature of the mice, which probably implies the body mass loss was due to an increase of energy expenditure not due to lower food intake." - Discussion.

Moreover, in this regard, our previous study determined that browning effect of SP on white adipose tissue in HFD-induced obese mice model. Please refer to this article [1]; Dietary Silk Peptide Prevents High-Fat Diet-Induced Obesity and Promotes Adipose Browning by Activating AMP-Activated Protein Kinase in Mice, Nutrients, 12(1), 2020, DOI: 10.3390/nu12010201.

4-The authors observed that fasting blood glucose was lower in SP treated mice and that these mice had an increase glucose tolerance compared to HF diet group.

These results suggest better insulin sensitivity or an increase insulin secretion. What about insulin levels? What about insulin sensitivity? Have you performed ITT?

Answer) Thank you for your suggestion. As your comment, our findings showed that SP may regulate insulin sensitivity or insulin secretion in HFD-induced obese mice. Even though we tried to ITT at the end of the experimental period, we could not analyze ITT due to technical problem, unfortunately. However, we suggest an article in Nutrients (in processing) to explain glucose homeostasis of SP in partial pancreatectomized rat model [2]. In this study, they also used same sample of SP obtained from Worldway Co., Ltd. and determined glucose level by fasting or intraperitoneal insulin tolerance test (IPITT) as well as insulin secretion. As a result, SP significantly reduced serum glucose concentrations in fasted and post-prandial states in Px rats to levels similar to those observed compared with control group. Also, their study indicates that SP dose-dependently not only reduced serum glucose but also elevated glucose-stimulated insulin secretion. Therefore, we added “The recent study reported that SP modulates the glucose level and insulin recreation in partial pancreatectomized rat moel, thereby SP may prevent non-obese T2D.” in discussion section.

Figure 3. Serum glucose levels during intraperitoneal insulin tolerance testing (IPITT) after a 6 h fast. A. Changes in serum glucose concentrations after an intraperitoneal injection of 1 IU of insulin/kg. Bars or dots and error bars represent means±SDs (n = 10). *indicates a significant intergroup difference (P<0.05). a,b,c Different letters on bars indicate significant differences (P<0.05) [2].

5-To determine whether SP administration had an anti-obesity effect the author analyzed adipose tissue.

What about fat mass? VAT weight?

Answer) As shown in Figure 1C, we presented the VAT weight of the mice treated with SP was significantly decreased. The reason why we measured VAT mass is to investigate whether VAT is
especially associated with anti-obesity effect and metabolic disease correlated glucose uptake capacity. However, we also observed the SAT, BAT, liver, lung, kidney, and spleen mass of the mice, so therefore added Table 1 to better understanding. Also, we revised Result 3.1 as exact explanation.

Table 1. Effect of SP treatment on organ weight in HFD-fed mice for 6 weeks.

<table>
<thead>
<tr>
<th>Organs</th>
<th>CD</th>
<th>HFD</th>
<th>HFD+SP50 *</th>
<th>HFD+SP200 *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visceral white adipose tissue (VAT)</td>
<td>1.42 ± 0.05c</td>
<td>3.74 ± 0.51a</td>
<td>3.03 ± 0.57b</td>
<td>1.57 ± 0.33c</td>
</tr>
<tr>
<td>Subcutaneous white adipose tissue (SAT)</td>
<td>0.74 ± 0.18c</td>
<td>1.67 ± 0.51a</td>
<td>1.43 ± 0.44b</td>
<td>0.95 ± 0.28c</td>
</tr>
<tr>
<td>Brown adipose tissue (BAT)</td>
<td>0.13 ± 0.03a</td>
<td>0.13 ± 0.03a</td>
<td>0.12 ± 0.04a</td>
<td>0.11 ± 0.02a</td>
</tr>
<tr>
<td>Liver</td>
<td>1.59 ± 0.21a</td>
<td>1.69 ± 0.15a</td>
<td>1.62 ± 0.32a</td>
<td>1.46 ± 0.14a</td>
</tr>
<tr>
<td>Lung</td>
<td>0.23 ± 0.03a</td>
<td>0.24 ± 0.02a</td>
<td>0.22 ± 0.02a</td>
<td>0.24 ± 0.02a</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.64 ± 0.08a</td>
<td>0.67 ± 0.07a</td>
<td>0.63 ± 0.08a</td>
<td>0.63 ± 0.04a</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.13 ± 0.02a</td>
<td>0.13 ± 0.02a</td>
<td>0.13 ± 0.02a</td>
<td>0.13 ± 0.06a</td>
</tr>
</tbody>
</table>

* (mg/kg/day). Data are expressed as mean ± SD (n = 6). Values with different letters are significantly different, p < 0.05 (a > b > c).

6-The authors claim that SP increases glucose absorption in fat tissue only on the basis of western-blot experiments.

Have you done glucose uptake experiments?

Answer) Thank you for your suggestion, but we could not assess glucose uptake by using the ELISA kit using 2-deoxyglucose due to the deficiency blood serum volume from the mice. However, our western data of VAT and skeletal muscle indicates that SP effectively increased the expression level of p-AMPK, p-AKT, p-IRS, and GLUT4. In addition we also estimated the effect of SP on HFD-induced hyperglycemia by oral glucose tolerance test (OGTT) and hemoglobin A1c (HbA1c) assay kit. GTT, known as the oral glucose tolerance test, is widely used to assess how body is able to absorb glucose after consuming a specific amount of sugar. Also, HbA1c test provides information about average of blood glucose level over the past 2-3 month. Therefore both of tests were used to check for diabetes or prediabetes in our mice model. Taken together, we suggest that SP has a potent to regulate glucose uptake in HFD-induced obese mice. Moreover, we suggest a patent regarding the anti-diabetes effect of SP from Worldway Co., Ltd. In this patent, it is explored that SP regulates glucose level in C57db/db mice model. Please refer to this patent-“A composition comprising peptide for treatment or prevention of diabetes mellitus, Publication number and date: 1020100020145 (05.03.2010)”.

7-If SP increases the absorption of glucose into the adipose tissue then you should have an increase in the amount of lipids in adipocytes. However, ORO staining in 3T3-L1 indicates that lipid accumulation in these cells was substantially prevented by SP.

How do you reconcile these two results?

Answer) As your advice, adipose tissue not only regulates lipid balance but also serves as a crucial integrator of glucose homeostasis. Also, lipid can be accumulated in early or middle differentiation of adipocytes, when glucose absorption was increased in adipocytes, as your advice. For instance, metformin is a widely used drug in the therapy of patients affected by diabetes mellitus and is a medication that many these people take to control blood glucose level by improved insulin sensitivity. However, metformin can induce fat accumulation again as an its side effect. In this regard, SP has a potent to regulate fasting glucose level without this side effect. In this study, we evaluated glucose uptake via GLUT4 expression in the mature differentiated 3T3-L1 for 8 days, thereby ORO staining data indicates the lipid accumulation after late-differentiated of adipocytes. In addition, we previously investigated that SP induced lipolysis and fatty acid oxidation by increasing the expression level of phosphorylated hormone-sensitive lipase (p-HSL), peroxisome proliferator-activated receptor alpha
(PPARα), and carnitine palmitoyltransferase 1 (CPT1) in VAT. Furthermore, we checked that SP induces WAT-to-BAT trans-differentiation by upregulating (Uncoupling protein 1) UCP1, thereby SP may dissipate fat to heat in adipocytes [1]. We revised this explain in discussion section.

8-Depending on the skeletal muscle studied, the contractile and metabolic characteristics are very different.

Which muscles did you use for the Western-block and immuno-fluorescence experiments?

Answer) We used gastrocnemius muscle of mice for skeletal muscle analysis in this study. Gastrocnemius muscle is one of the large muscles of the leg, and this muscle is associated with out grip strength test data. Regarding on it, we have conducted experiments such as western blot and immusostaining analysis with reference to many papers, reported the effect of various dietary materials on gastrocnemius muscle in obese mice [3, 4]. Therefore, we correctly wrote the name of the muscle in manuscript.

9-Skeletal muscle is characterized by its mass, contractile type and metabolic activity.

What is the mass of muscles such as the quadriceps, gastrocnemius, tibialis?

Are there changes in the expression of myosin heavy chains?

Are there changes in the activity of mitochondrial respiratory chain complexes?

Answer) To study skeletal muscle physiology, we wanted to isolate the muscle separate the gastrocnemius, tibialis anterior, and soleus muscle from the HFD-fed obese mice. Since it was our first time to research the muscle or sarcopenic obesity, so therefore only gastrocnemius muscle can be analyzed with the help of other researchers. We also feel sorry for this situation and please understand it.

Instead, we suggest some data added as a supplementary data, which is observed before this present study, as your kind advice. We estimated that whether SP administration for 8 weeks enhances the muscle size and strength in 12-month-old male C57BL/6J mice, compared with 6-week-old male C57BL/6J mice. At that time, since appropriate concentration of SP administration was not decided yet, we provided to mice with 250 mg/kg/day of SP. According to our supplementary data, SP restores age-related declines in mass of hindlimb muscle. Also, age-related decreases in muscle strength were increased by SP. While body mass of OM+SP200 has no significant difference by SP treatment, it was slightly decreased compared OM.

Supplementary data 1. Effect of SP on muscle size and strength, and body weight in old mice. (A) Micro-CT image of hindlimb muscle. (B) Relative hindlimb muscle volumes were quantitated
compared with those of 0 week. (C) Latency of forced swimming test was measured for 30 min. (D) Body weight of the mice after 8 weeks of experimental period. *p < 0.05, **p < 0.01 compared to young mice group at 0 week; †p < 0.05, ‡p < 0.01 compared to old mice group at 0 week.

Also, as your comment, skeletal muscles consist of various myosin heavy chain (MyHC). Therefore we investigated the enhancing effect of SP on muscle fiber area by using western blot and immunostaining. The antibody of MyHC used in this study was “Anti-heavy chain Myosin/MYH3 antibody (ab124205)” and it was purchased from Abcam. We revised manuscript as “In addition, H&E-stained images and MyHC immunostaining data of gastrocnemius muscle (Fig. 6B) show that SP treatment dose-dependently increased fiber cross-section area- which implies that SP encourages myogenic differentiation.” in result section.

Lastly, we explored the mitochondrial activity in gastrocnemius muscle of obese mice. Recent research reported that muscle mitochondrial-related genes including NRF1, PGC1α and UCP3 regulates insulin resistance during obesity [5, 6]. Especially, PGC1α is well-known transcription factor correlated with sarcopenia and metabolic disease during aging [7, 8]. Therefore, our finding indicates that SP increased the expression level of these protein implies SP may increase the mitochondrial respiration in skeletal muscle in HFD-induced obese mice.

10-In figure 6, the authors claim that SP treatment increased fiber cross-section area.

If the authors have made analyses of the whole section area and quantified the number and surface area of the fibers it would be nice to show this.

Answer) As your advice, we revised the Figure 6 with quantitative data of muscle fiber area (%). We also added the information in figure legend as “(B) Hematoxylin and eosin staining and immunofluorescence staining of MyHC in muscle from mice treated for 6 weeks. Its relative average area of 20-25 muscle fiber was quantified per same area using Image J software.”

11-Furthermore, the authors claim that SP treatment increased MyHC expression in HF diet. This statement is nonsense. The muscle remains muscle and it is mostly composed of MyHC. Their antibody is probably directed against a type of myosin and their western-blot suggest rather that SP induces a change in the contractile type.

Which isoform of MyHC recognizes your antibody?

Answer) As we answered n Q6, skeletal muscles consist of various myosin heavy chain (MyHC). Therefore we investigated the enhancing effect of SP on muscle fiber area by using western blot and immunostaining. The antibody of MyHC used in this study was “Anti-heavy chain Myosin/MYH3 antibody (ab124205)” and it was purchased from Abcam. We revised as “In addition, H&E-stained images and MyHC immunostaining data of gastrocnemius muscle (Fig. 6B) show that SP treatment dose-dependently increased fiber cross-section area- which implies that SP encourages myogenic differentiation.” in result section. We appreciate that your suggestion

12-It is essential that you indicate the exact references of the antibodies used. For example, some phosphorylations activate AKT while others inhibit it. The interpretation is not the same...

Answer) As reviewer’s comment, we added phosphorylation site of each factor in “2.1 Materials” on page 3 line 100-102, as “Anti-C/EBPα, PPARγ, myogenin, AKT, p-AKT (Ser 473), AMPK, and p-AMPK (Thr 172) antibodies were obtained from Cell Signaling Technology (Danvers, MS, USA). Anti-DGAT1, GAPDH, Fbx32, IRS, and p-IRS (Thr 632) antibodies were purchased from Santa Cruz Biotechnology, CA, USA.”
13-The authors claim that SP may prevent sarcopenia by enhancing muscle differentiation and inhibiting the muscle atrophy induced by HF diet.

Influence of SP on muscle Weight? Have you studied autophagy or synthesis flux? Have you studied the influence of SP on skeletal muscle regeneration?

Answer) As we answered in Q9, we observed that SP prevents age-related sarcopenia by enhancing muscle endurance. Although we could not measure the weight of each muscle of HFD-induced obese mice, our data indicates that SP may prevent sarcopenia by examining the expression levels of E3-ubiquitinase factors (MuRF1 and Fbx32), mitochondrial biogenesis related factors (UCP3, NRF1 and PGC1α), and myoblast differentiation factors (MyHC and Myogenin) in skeletal muscle of HFD fed mice. As your kind suggestion, even though we have to study autophagy or synthesis flux of muscle in further, please understand we was first time to study on muscle dystrophy. Reviewer’s kind advices will be helped us in setting our further research.

14-The authors claim that SP stimulates myoblast differentiation.

Did you measure the fusion index to state this?

Answer) As reviewer’s advice, we revised Figure 7 with fusion index (%). To estimate the percentage fusion, we calculated at the end of differentiation by dividing the number of nuclei within multinucleated myofibers by the total number of nuclei.

References


