Respond to Reviewers' Comments

Editor in Chief

We are pleased to inform you that, after preview by the Editorial Office and peer review as well as CrossCheck and Google plagiarism detection, we believe that the academic quality, language quality, and ethics of your manuscript (Manuscript NO.: 76727, Basic Study) basically meet the publishing requirements of the World Journal of Stem Cells. As such, we have made the preliminary decision that it is acceptable for publication after your appropriate revision.Upon our receipt of your revised manuscript, we will send it for re-review. We will then make a final decision on whether to accept the manuscript or not, based upon the reviewers' comments, the quality of the revised manuscript, and the relevant documents.

Answer: Thanks for all the suggestions. We have checked our manuscript with full professional proficiency according to the editorial policy of this journal, in which the answers for the reviewer's questions are attached as "Respond to Reviewers' Comments".

Reviewer 1

The manuscript by Li et al., was a relatively comprehensive study on the roles of the genes regulations of the bone marrow cells. The authors seemed to focus on the stem cell differentiation and tested the molecular and cellular changes in the obesity of the mice. Experiments were well performed, and data was well collected. Due to experimental design issues, the article might be more suitable for more specific journals. There were some significant concerns:

1. The evaluation of the normal development should be considered.

Answer: Thanks for your suggestion. We have already added this section in the Results (Among the male offspring, their body weight and mental state at weaning were not significantly different among the NC-C, DC-HFD, LC-HFD, NC-HFD and HC-HFD groups (Figure 1A)). Besides, our previous studies also had confirmed that maternal NC, LC, and HC intake groups did not affect the normal development of their offspring.

Reference:

[1] Li P, Chang X, Fan X, Fan C, Tang T, Wang R, Qi K. Dietary calcium status during maternal pregnancy and lactation affects lipid metabolism in mouse offspring. Sci Rep. 2018;8(1):16542.

[2] Li P, Tang T, Chang X, Fan X, Chen X, Wang R, Fan C, Qi K. Abnormality in Maternal Dietary Calcium Intake During Pregnancy and Lactation Promotes Body Weight Gain by Affecting the Gut Microbiota in Mouse Offspring. Mol Nutr Food Res. 2019;63(5):e1800399.

[3] Li P, Yan K, Chang X, Chen X, Wang R, Fan X, Tang T, Zhan D, Qi K. Sex-specific maternal calcium requirements for the prevention of nonalcoholic fatty liver disease by altering the intestinal microbiota and lipid metabolism in the high-fat-diet-fed offspring mice. Gut Microbes. 2020;11(6):1590-1607.

2. Calcium-dependent genes in diseases should be referenced (such as DOI: 10.3389/fpsyt.2020.00080).

Answer: Thanks for your comments. Previously, we had not found any significant results of the calcium-dependent genes among maternal different calcium intake groups (DC, LC, NC and HC), which could be caused by the same dietary with normal contents of calcium after weaning.

3. The fat deposition was mentioned (such as in Discussion section Lines 419-420 but not directly tested/measured.

Answer: Thanks for your comments. We have already modified this section (Lines 419-420) in the Discussion as following: maternal calcium dysfunction could directly affect the **fat synthesis and metabolism** of their offspring, which come from the studies performed in the animal models, which were also proved in our results.

4. The "lineage-specific commitment" (Line 440) and related concept were logical unclear.

Answer: Thanks for your advice. We have already modified this section (Line 440) in the Discussion according to the references as following: lineage-tracing studies in the animal models suggested that there were two-step phases in the adipogenic differentiation, including the specific preadipocyte formation (from MSCs to preadipocyte) and terminal adipocyte maturation (from preadipocyte to mature adipocytes).

Reference

[44] Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, Spiegelman BM, Mortensen RM. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Mol Cell 1999; 4: 611-7.

[45] Farmer SR. Transcriptional control of adipocyte formation. Cell Metab 2006; 4: 263-73.

5. How the other MSCs (from different origins) were affected should be studied.

Answer: Thanks for your criticism. Firstly, as the reference, there are many types of MSCs according to their different sources in the C57BL/6 mice, including the umbilical cord mesenchymal stem cells (ucMSCs), placental mesenchymal stem cells (pMSCs), bone marrow mesenchymal stem cells (BMSCs) and adipose stromal cells (ADSCs), in which the BMSCs and ADSCs are main MSCs by considering the convenience of obtaining the biological materials. However, the dietary intervention in this study is before the weaning, so we chose the BMSCs as the mainly results. Secondly, we have already added this limitation in the Discussion (Furthermore, there were still some limitations in this study: Firstly, it requires a more complicated and explicit mechanism procedure including the Western blot. Secondly, this conclusion should be verified in the other MSCs and other animal models to ensure its feasibility and effectiveness).

6. The study can at least describe the female offspring scientifically.

Answer: Thanks for your suggestion. We have already added this section in the Methods. The reasons that we only selected the male offspring as the experimental subjects were as follows:

Firstly, the male mice, not the female mice, are routinely used to study the metabolic diseases such as the development of obesity.

Reference:

[1] Xu L, Nagata N, Chen G, Nagashimada M, Zhuge F, Ni Y, Sakai Y, Kaneko S, Ota T. Empagliflozin reverses obesity and insulin resistance through fat browning and alternative macrophage activation in mice fed a high-fat diet. BMJ Open Diabetes Res Care. 2019;7(1):e000783.

[2] Le Roy T, Moens de Hase E, Van Hul M, Paquot A, Pelicaen R, Régnier M, Depommier C, Druart C, Everard A, Maiter D, Delzenne NM, Bindels LB, de Barsy M, Loumaye A, Hermans MP, Thissen JP, Vieira-Silva S, Falony G, Raes J, Muccioli GG, Cani PD. Dysosmobacter welbionis is a newly isolated human commensal bacterium preventing diet-induced obesity and metabolic disorders in mice. Gut. 2022;71(3):534-543.

[3] Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444(7122):1027-31.

Secondly, our previous study had proved that "there is a sex-specific manner for maternal calcium requirement to inhibit the progress of offspring NAFLD, that might be less for the female offspring (0.25%) and more for the male offspring (0.70%)". So the existing animal model is feasible for the male offspring (0.70%-NC group). However, the classification of these groups with different dietary calcium intake (DC, LC, NC and HC groups) is inappropriate for the female offspring, which still needs to be further discussed in the future.

Our study:

[1] Li P, Yan K, Chang X, Chen X, Wang R, Fan X, Tang T, Zhan D, Qi K. Sex-specific maternal calcium requirements for the prevention of nonalcoholic fatty liver disease by altering the intestinal microbiota and lipid metabolism in the high-fat-diet-fed offspring mice. Gut Microbes. 2020;11(6):1590-1607.

7. It is noted that the weaning may affect the results. It needs to be solved.

Answer: Thanks for your criticism. Firstly, we will try to explore the impacts of different calcium interventions during the pregnancy and after weaning on the health of their male offspring in the future. Then, the aim of our study was to present the efficient possibility to derive the hyperplasic adipogenesis from bone mesenchymal stem cells (BMSCs) by regulating the gene expression profiles through to adulthood. In our study, the male offspring were with the same feeding and dietary environments except that only the dietary calcium intake during the pregnancy was inconsistent, which might verify the aim of this study and play a significant role on providing certain scientific data for the human researches.

8. In the BMSCs experimental results in Table 1 and related design, cells need to be characterized. The statistics is unclear, and it may need re-analyzing.

Answer: Thanks for your suggestion. The BMSCs have been characterized in both **Table 1** and **Figure S1**, in which 1×10^6 BMSCs were used in the DC, LC, NC and HC groups to measure the percentage of BMSCs with the related antibodies(Sca-1, CD90, CD31, CD29, CD34, CD45, and CD49d). Besides, all these above results were re-analyzed in the Methods section by the χ^2 test .

Reviewer: 2

I would like to congratulate the authors for this manuscript. The study is interesting and brings new perspective. I have some comments about the manuscript:

1. Materials and methods/experimental procedures: Please add brief description or reference from previous study (if any) regarding the calcium percentage determination in the reproductive diets (0.05%, 0.25%, 0.70%, 1.20%) in relation to table 2 (the diet formula) for better understanding.

Answer: Thanks for your suggestion. We have already added this contents in the Table 1.

Both previous and our researches in the animal models had shown that dietary calcium concentrations were ranged from 0.4% to 1.0% to promote the normal growth and development. Besides, the dietary calcium at a concentration of 0.02% would cause the delay on the growth. For the diets we used, total calcium contents were 0.25%, 0.7% and 1.2% respectively in the low-, normal- and high- calcium diets, including 1.8, 6.3 and 11.4 g of calcium added and calcium in the casein respectively. Furthermore, we used the 0.7% calcium as normal diet, which was slightly higher than 0.57% in the breeding AIN-93G diet. Thus, the calcium concentration at 0.25% in the LC diet represented an insufficient level instead of deficient for typical breeding diets, while its concentration at 1.2% in the HC diet was calcium-excessive. And the calcium in the casein was 0.05% as the deficient- calcium diet.

Reference

[1] Chaplin A, Parra P, Laraichi S, Serra F, Palou A. Calcium supplementation modulates gut microbiota in a prebiotic manner in dietary obese mice. Mol Nutr Food Res. 2016; 60(2): 468-80.

[2] Gomes JM, Costa JA, Alfenas RC. Could the beneficial effects of dietary calcium on obesity and diabetes control be mediated by changes in intestinal microbiota and integrity? Br J Nutr. 2015; 114(11): 1756-65.

[3] Li P, Yan K, Chang X, Chen X, Wang R, Fan X, Tang T, Zhan D, Qi K. Sex-specific maternal calcium requirements for the prevention of nonalcoholic fatty liver disease by altering the intestinal microbiota and lipid metabolism in the high-fat-diet-fed offspring mice. Gut Microbes. 2020;11(6):1590-1607.

[4] Li P, Tang T, Chang X, Fan X, Chen X, Wang R, Fan C, Qi K. Abnormality in Maternal Dietary Calcium Intake During Pregnancy and Lactation Promotes Body Weight Gain by Affecting the Gut Microbiota in Mouse Offspring. Mol Nutr Food Res. 2019 Mar;63(5):e1800399.

2. Please add the rats' initial maternal body weight for all the groups that were used in the study.

Answer: As shown in the **Figure S1-A** and **Methods,** the four weeks old C57BL/6N female mice were obtained from Beijing Vital River Laboratory Animal Technology and housed at the animal facilities in the laboratory animal center of Academy of Military Medical Sciences **(As the 0.0 month in the Figure S1-A)**. The mice were randomly divided into four groups and fed with the reproductive deficient (0.05%), low (0.25%), normal (0.70%) and high-calcium (1.20%) diets respectively for 6 weeks, then the remaining female mice were mated with 10-week-old C57BL/6N male mice to create their male offspring **(As the 1.5 months in the Figure S1-A)**. The body weights were not significant differences among the four groups from the initial feeding to 6 weeks (1.5 months).

Maternal body weight for all the groups

Months	0.05%	0.25%	0.70%	1.20%	*P*
0.0	12.38±0.77	12.72±1.38	12.43±0.82	12.43±0.80	0.985
0.5	17.55±0.86	17.27±0.71	17.40±0.43	17.45±0.48	0.904
1.0	18.72±0.78	19.50±1.00	18.37±0.88	19.12±0.26	0.756
1.5	19.65±0.86	20.65±0.56	20.08±0.36	20.00±0.65	0.342

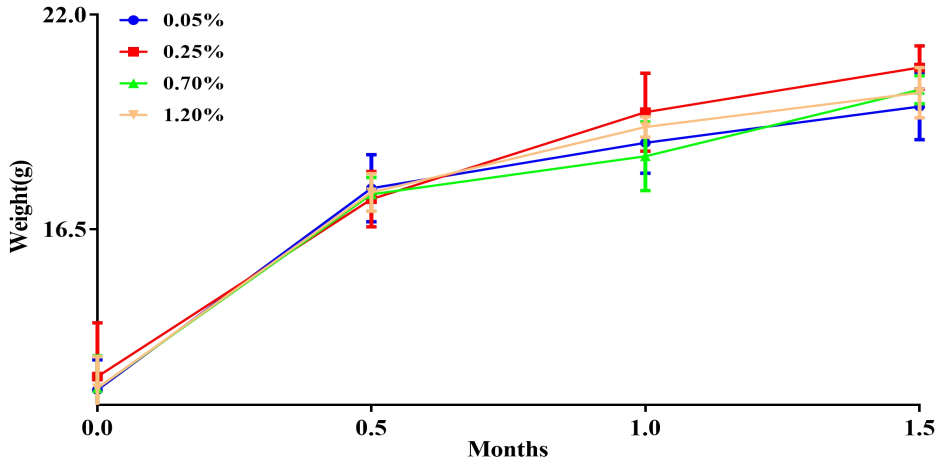

Figure S1-A. Effects of different dietary calcium intake on the body weight among the mother mice.

3. Line 148: "the low-fat diet", does this supposed to be "the normal diet" as the control group (NC-C)? please clarify.

Answer: Thanks for your advice. We have already modified the Line 148 in the Experimental Procedures section: the normal diet (4.3% fat by weight, 10% kcal, No. H10010).

4. Line 151: For animal termination, was inhalation of carbon dioxide the only method used or was additional anesthetic agent administered to the rats? if yes please clarify.

Answer: Thanks for your advice. We have already modified the Line 151 in the Experimental Procedures section: The blood samples were collected through the eye-drop, then they were inhalation of carbon dioxide to minimize the animal pain. So all mice were inhalation of carbon dioxide as the only method used in this study.

5. Line 152: please clarify "blood samples were collected through eye-drop to minimize their suffering under the 12-hour fasting", does this means the blood samples were collected retro-orbitally? or other method was used, please specify.
Answer: Thanks for your criticisms. We have already modified this section in the manuscript: The blood samples were collected through the eye-drop, then they were inhalation of carbon dioxide to minimize the animal pain.

6. Please make sure to use SI units according to guidelines for authors.
Answer: Thanks for your advice. We have already modified the SI units according to guidelines for authors.

7. There are several mistyped words and lack of space between words in several sentences, please double check thoroughly. Mistyping of "resistin" are found in line 333, 341, 342, 343, 345, 447, 453, and 507. Line 153: rpm/min does it means r/min? please make sure there are no mistyped words.
Answer: Thanks for your criticism. We have already modified all the mistyped words in the whole manuscript, including the "resistin" and “r/min”.

Reviewer 3

1. Why were male offspring selected as experimental subjects?
Answer: Thanks for your criticism. The reasons that we selected the male offspring as the experimental subjects were as follows:

Firstly, the male mice, not the female mice, are routinely used to study the metabolic diseases such as the development of obesity.

Reference:

[4] Xu L, Nagata N, Chen G, Nagashimada M, Zhuge F, Ni Y, Sakai Y, Kaneko S, Ota T. Empagliflozin reverses obesity and insulin resistance through fat browning and alternative macrophage activation in mice fed a high-fat diet. BMJ Open Diabetes Res Care. 2019;7(1):e000783.

[5] Le Roy T, Moens de Hase E, Van Hul M, Paquot A, Pelicaen R, Régnier M, Depommier C, Druart C, Everard A, Maiter D, Delzenne NM, Bindels LB, de Barsy M, Loumaye A, Hermans MP, Thissen JP, Vieira-Silva S, Falony G, Raes J, Muccioli GG, Cani PD. Dysosmobacter welbionis is a newly isolated human commensal bacterium preventing diet-induced obesity and metabolic disorders in mice. Gut. 2022;71(3):534-543.

[6] Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444(7122):1027-31.

Secondly, our previous study had proved that "there is a sex-specific manner for maternal calcium requirement to inhibit the progress of offspring NAFLD, that might be less for the female offspring (0.25%) and more for the male offspring (0.70%)". So the existing animal model is feasible for the male offspring (0.70%-NC group). However, the classification of these groups with different dietary calcium intake (DC, LC, NC and HC groups) is inappropriate for the female offspring, which still needs to be further discussed in the future.

Our study:

[1] Li P, Yan K, Chang X, Chen X, Wang R, Fan X, Tang T, Zhan D, Qi K. Sex-specific maternal calcium requirements for the prevention of nonalcoholic fatty liver disease by altering the intestinal microbiota and lipid metabolism in the high-fat-diet-fed offspring mice. Gut Microbes. 2020;11(6):1590-1607.

2. The experiment uses NC as the control group. It is better to compare the NC, DC, LC, and HC groups with each other and discuss their differences.

Answer: Thanks for your suggestion. We have already compared the differences in the NC, DC, LC, and HC groups with each other and added this contents in the Discussion. Besides, our previous studies also had confirmed that the differences among the NC, DC, LC, and HC groups were more obvious after high fat induction, so we mainly described these contents in our manuscript according to the results in our previous references.

Reference:

[1] Li P, Chang X, Fan X, Fan C, Tang T, Wang R, Qi K. Dietary calcium status during maternal pregnancy and lactation affects lipid metabolism in mouse offspring. Sci Rep. 2018;8(1):16542.

[2] Li P, Tang T, Chang X, Fan X, Chen X, Wang R, Fan C, Qi K. Abnormality in Maternal Dietary Calcium Intake During Pregnancy and Lactation Promotes Body Weight Gain by Affecting the Gut Microbiota in Mouse Offspring. Mol Nutr Food Res. 2019;63(5):e1800399.

[3] Li P, Yan K, Chang X, Chen X, Wang R, Fan X, Tang T, Zhan D, Qi K. Sex-specific maternal calcium requirements for the prevention of nonalcoholic fatty liver disease by altering the intestinal microbiota and lipid metabolism in the high-fat-diet-fed offspring mice. Gut Microbes. 2020;11(6):1590-1607.

3. Only the results of qPCR cannot fully prove the changes of adipogenic differentiation, osteogenic differentiation and Wnt pathways. It is recommended to supplement the Western blot results of key genes.

Answer: Thanks for your suggestion. It is the most important limitation in our research, then we have done the following improvements to make up this limitation as much as possible.

Firstly, we have already modified the Conclusion in our manuscript to demonstrate that there were certain correlations between the adipogenic differentiation and related gene expression of many genes in the Wnt/β-catenin signaling pathway. Conclusion section: These above results suggested that dietary

abnormal calcium intake in early life might program the adipogenic differentiation potential of BMSCs, which was related with the abnormal expression of many genes in the Wnt/β-catenin signaling pathway to reserve more preadipocytes.

Meanwhile, it has been found that the mRNA expressions in the genes on the adipogenic proliferation, differentiation and Wnt/β-catenin signaling pathway are accompanied with the changes of their expressions using the Western blots according to the existing literature, by which the conclusion of this study is meaningful and credible. In this condition, the specific mechanism especially the Western blot results of key genes will need to be further explored.

Reference:

[1] Deng Q, Li P, Che M, Liu J, Biswas S, Ma G, He L, Wei Z, Zhang Z, Yang Y, Liu H, Li B. Activation of hedgehog signaling in mesenchymal stem cells induces cartilage and bone tumor formation via Wnt/β-Catenin. Elife. 2019;8:e50208.

[2] Rong X, Liu J, Yao X, Jiang T, Wang Y, Xie F. Human bone marrow mesenchymal stem cells-derived exosomes alleviate liver fibrosis through the Wnt/β-catenin pathway. Stem Cell Res Ther. 2019;10(1):98.

[3] El-Derany MO, Said RS, El-Demerdash E. Bone Marrow-Derived Mesenchymal Stem Cells Reverse Radiotherapy-Induced Premature Ovarian Failure: Emphasis on Signal Integration of TGF-β, Wnt/β-Catenin and Hippo Pathways. Stem Cell Rev Rep. 2021;17(4):1429-1445.

[4] Elbaz EM, Helmy HS, El-Sahar AE, Saad MA, Sayed RH. Lercanidipine boosts the efficacy of mesenchymal stem cell therapy in 3-NP-induced Huntington's disease model rats via modulation of the calcium/calcineurin/NFATc4 and Wnt/β-catenin signalling pathways. Neurochem Int. 2019;131:104548.

Finally, the expressions of existing genes on the adipogenic proliferation, differentiation and Wnt/β-catenin signaling pathway in the BMSCs and adipose tissues were still unclear. However, we recently had not found any consistent and significant results in our experiments for their low expressions. Meanwhile, we still tried to determine the expressions of key genes (*PPARγ, C/EBPα, Runx2, CTNNB1, Wnt5a* and *Gsk3β*) using the Western blots by improving the experimental conditions and antibody brands. Sincerely, we hope that we can further analyze and deal with this limitation in the future, which could be communicated and exchanged with you by email.

4. Please reflect the second part of the results-"Maternal abnormal dietary calcium intake could cause the disorders of the infiltration of immune cells in the adipose tissues among their male offspring" in the discussion.

Answer: Thanks for your advice. We have already added the second part of the results in the Discussion as our previous study.

[1] Li P, Li P, Liu Y, Liu W, Zha L, Chen X, Zheng R, Qi K, Zhang Y. Maternal vitamin D deficiency increases the risk of obesity in male offspring mice by affecting the immune response. Nutrition. 2021; 87-88:111191.