Dear Editor,

We are pleased to resubmit for publication in your journal the revised version of Manuscript (NO. 93244). We really appreciated the suggestions from you and the reviewer, which we have tried to address.

Following your comments throughout the manuscript, we made the following changes and additions which have been highlighted (in Yellow) in the revised manuscript word file:

1. We revised all issues that are raised in the peer-review report and provide point-by-point responses to each of the issues raised in the peer-review report.
2. The revised version has been edited carefully by native English language expert to ensure that the language is clear and free of errors throughout the manuscript.
3. We revised all Figures and Figure legends according to the "Checklist for Authors to Revise a Manuscript", which have been placed in separate pages at the end of the revised manuscript.
4. We prepared and arranged the figures using PowerPoint, and submitted those original figure documents to F6Publishing.
5. We revised all statistical significance to superscript letters, such as \(^a P < 0.05\), \(^b P < 0.01\), etc.
6. We edited the references using the Auto-Analyser in F6Publishing to ensure the correctness of all reference information.
7. We added the "Core Tip" as below.

"This study demonstrated interactions between myoblasts and macrophages under high glucose (HG) milieu induced pro-inflammatory M1 polarization of macrophages to exacerbate inflammatory response. Subsequently, chronic inflammation induced by HG-related M1 macrophages damaged myogenesis
and insulin sensitivity in myoblasts. Ultimately, interactions between myoblasts and macrophages resulted in skeletal muscle insulin resistance (IR), which supported macrophage may serve as a promising therapeutic target for skeletal muscle atrophy and IR. This is the first research about the mediation of macrophages to HG-related myogenic inhibition and IR in myoblasts, which provide new insights into the prevention and treatment of skeletal muscle atrophy and IR.

The responses to reviewers are listed below.

Thank you very much for your consideration. We look forward to hearing from you.

Sincerely,
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To follow, answers to the reviewer:

Dear reviewer,

Thank you for carefully reading this manuscript and for providing suggestions and comments. The manuscript has benefited a lot from these insightful suggestions.

Please find a point-by-point response to your comments:

Specific Comments to Authors:
I have now reviewed your paper and recognize the importance of your research question. Manuscript NO. 93244 aimed to assess the interaction dynamics between myoblasts and macrophages in environments with elevated glucose levels, specifically focusing on their effects on inflammatory response and insulin sensitivity within skeletal muscle.

1. The “Abstract” needs improvements.
   a. “BACKGROUND” — Further exploration of macrophages' role in insulin resistance (IR) and the dynamics of their infiltration and polarization is warranted. Additionally, the omission of myoblasts weakens the study's objective.

   **Revision**: Thank you for your suggestion. In the revised version, we rewrote “BACKGROUND” of “Abstract” section and added myoblasts relative statement:
   
   "Skeletal muscle handles about 80% of insulin-stimulated glucose uptake and become the major organ occurring insulin resistance (IR). Many studies have confirmed the interactions between macrophages and skeletal muscle regulated the inflammation and regeneration of skeletal muscle. However, despite decades of research, whether macrophages infiltration and polarization in skeletal muscle under high glucose (HG) milieus results in the development of IR is yet to be elucidated. C2C12 myoblasts are well-established and excellent model to study myogenic regulation and its responses to stimulation. Further exploration of macrophages' role in myoblasts IR and the dynamics of their infiltration and polarization is warranted".

   b. The “RESULTS” are presented succinctly, but it would be helpful to include some quantitative data or statistical information to support the findings and strengthen the impact of the study.

   **Revision**: Thank you for your reminding. In the revised version, we added statistical information to the “RESULTS” of “Abstract” section:
"The F4/80 and co-localization of F4/80 and CD86 increased, and the myofiber size decreased in IR group (p < 0.01, g = 6.26). Compared to Mc group, F4/80+CD86+CD206- cells, TNFα, IL-1β and IL-6 decreased, and IL-10 increased in McM group (p < 0.01, g > 0.8). In McM+HG group, F4/80+CD86+CD206- cells, MCP1, TNFα, IL-1β and IL-6 increased, and F4/80+CD206+CD86- cells and IL-10 decreased compared both with Mc+HG group and McM group. Compered to M group, myotube area, myotube number and E-MHC increased in MMc group (p < 0.01, g > 0.8). In MMc+HG group, myotube area, myotube number, E-MHC, GLUT4 and glucose uptake decreased compared with M+HG group and MMc group (p<0.01, g > 0.8) ".

c. “CONCLUSION” — While the study’s potential impact on understanding insulin resistance is mentioned, it could be helpful to explicitly state how these findings could inform future research or clinical interventions in the field. Additionally, discussing any limitations or future directions for research would add depth to the conclusion.

**Revision:** Thank you for your suggestion. In the revised version, we rewirited “CONCLUSION” of “Abstract” section and stated how these findings could inform future research and clinical interventions:

“Interactions between myoblasts and macrophages under HG milieus result in inflammation and IR, which support macrophage may serve as a promising therapeutic target for skeletal muscle atrophy and IR”.

Because the journal requires no more than 30 words in “CONCLUSION” of “Abstract” section, we discussed research limitations in "DISCUSSION" section:

"However, this study has certain limitations. Firstly, in order to really demonstrate the effects on inflammation and IR in this study are just related to glucose per se and not to hyperosmolarity induced by 60 mmol/L glucose, we used L-glucose as an osmotic control for 60 mmol/L D-glucose. So, future researches maybe needed to analyze the damage of hyperosmolarity caused by HG to inflammation and insulin sensitivity. Further, considering the sample
size (n = 6 per group) may be relatively small in this study, more samples would be needed for further research".

d. LANGUAGE AND GRAMMAR: There are several instances of grammatical errors and awkward phrasing throughout the Abstract.

**Revision:** Thank you for your reminding. The revised version has been edited carefully by native English language expert to ensure that the language is clear and free of errors throughout the manuscript.

2. Overall, the INTRODUCTION section presents the significant points related to the research background and objectives; however, it could benefit from further emphasis on clinical-epidemiological aspects to strengthen its relevance.

**Revision:** Thank you for your suggestion. In the revised version, we further emphasised clinical-epidemiological aspects to strengthen its relevance in the INTRODUCTION section:

"The prevalence of diabetes, especially type 2 diabetes mellitus (T2DM), has been dramatically increasing in China, from 10.9% in 2013 to 12.4% in 2018. Insulin resistance (IR) is considered the pathogenic driver of T2DM and precedes non-physiologic elevated plasma glucose levels, which is the primary clinical symptom of T2DM. In the prediabetic condition, insulin levels increase to meet normal insulin requirements leading to chronic hyperinsulinemia, hyperglycemia-induced β-cell failure, and eventually to T2DM [1, 2]."

**Reference**


2 **Ortiz GG**, Huerta M, González-Usigli HA, Torres-Sánchez ED, Delgado-Lara DL, Pacheco-Moisés FP, Mireles-Ramírez MA, Torres-Mendoza BM, Moreno-Cih RI, Velázquez-Brizuela IE. Cognitive disorder and dementia in
3. METHODS:

(1) Please mention the rationale behind the choice of glucose and insulin doses in both in vivo and in vitro experiments.

**Revision**: Thank you for your reminding. In the revised version, we added the rationale behind the choice of glucose doses in the DISCUSSION section:

"In addition, 25 mmol/L glucose is recommended by the American Type Culture Collection for myoblasts culture, so DMEM with 25 mmol/L glucose should be regarded as the norm caloric conditions [44]. For the high glucose milieu, we originally treated macrophages and myoblasts with 40 mmol/L or 60 mmol/L glucose. By preliminary experiments, we found that 40 mmol/L glucose had a slight but non-significant effect on macrophages polarization and myoblasts differentiation, but 60 mmol/L glucose significantly induced M1 macrophages and inhibited myoblasts differentiation. Since the main aim of this study is to explore the potential mechanisms of high glucose inducing inflammatory response and myoblasts IR, the glucose dosage that significantly effected macrophages polarization and myoblasts differentiation should be used. So, in this study, we used 60 mmol/L glucose as the high glucose condition."

For insulin dose, the papers below have been added to Reference (reference #35-36) as the rationale for the selection of 100 nmol/L:” To detect the insulin sensitivity of myotube, C2C12 cells were incubated in the absence or presence of insulin (100 nmol/L) for 30 min at 37 °C before gathered [35,36]."

**Reference**


(2) Additionally, highlighting the sensitivity and specificity of the ELISA kits utilized holds significance.

**Revision:** Thank you for your suggestion. In the revised version, we added the sensitivity and specificity of the ELISA kits in the “Enzyme Linked Immunosorbent Assay (ELISA) for culture medium” subsection:

"The protein levels of monocyte chemoattractant protein 1 (MCP1, MJE00B, R&D, USA, sensitivity: 0.666 pg/mL, specificity: mouse), tumor necrosis factor-α (TNFα, MTA00B, R&D, USA, sensitivity: 7.21 pg/mL, specificity: mouse), Inerleukin-1β (IL-1β, MLB00C, R&D, USA, sensitivity: 4.8 pg/mL, specificity: mouse), IL-6(M6000B, R&D, USA, sensitivity: 1.8 pg/mL, specificity: mouse), and IL-10 (M1000B, R&D, USA, sensitivity: 5.22 pg/ml, specificity: mouse) secreted into medium was quantified with ELISA kit according to the manufacturer’s instructions".

(3) Regarding statistical analysis, methods employed to address deviations from normality should be described.

**Revision:** Thank you for your reminding. In this research, we calculated p > 0.05 in each group by the Shapiro-Wilk test, which indicated the data in this research followed a normal distribution. So, the use of two-tailed Student’s t-test and two-way ANOVA test for statistical analysis in this research may be adequate.

In the revised version, we have added the relevant statements:

"According to the Shapiro-Wilk test, the data in each group followed a normal distribution".
It is currently considered crucial to report effect sizes for group differences. Please include methods for effect size estimation in the “Statistical analysis” subsection.

**Revision:** Thank you for your reminding. In the revised version, we added the methods for effect size estimation in the “Statistical analysis” subsection:

"Hedges’ g effect sizes were calculated, and effect sizes were interpreted as: $g < 0.2$ very small, $g = 0.2 - 0.5$ small, $g = 0.5 - 0.8$ medium, $g > 0.8$ large."

4. RESULTS: Overall, the study offers evidence connecting insulin resistance with inflammatory mechanisms in skeletal muscle, specifically implicating macrophage infiltration, M1 polarization, and cytokine release. However, certain areas for improvement could bolster the reliability of the results.

(1) While the use of two-tailed Student’s t-test and two-way ANOVA for statistical analysis is appropriate, the study would benefit of effect size reporting for group differences.

**Revision:** Thank you for your reminding. In the revised version, we have added the effect sizes in the RESULTS section.

(2) Moreover, the sample size ($n = 6$ per group) may be small for drawing generalizable conclusions, particularly in complex biological systems.

**Revision:** Thank you for your reminding. The sample size ($n = 6$ per group) is indeed relatively small. But the same with most researches in which the sample sizes are 3-6 per group [1-3], this sample size may be workable to draw reliable and generalizable conclusions. Despite all this, your suggestion gives us a nice reminder, we will increase sample size in the future study to make our results and conclusion generalizable more.

In the revised version, the relative statement has been added to DISCUSSION section as the limitations of this study:

"Further, considering the sample size ($n = 6$ per group) may be relatively small in this study, more samples would be needed for further research".
Reference:


5. The DISCUSSION scrutinizes the findings through systematic theoretical analyses. However, there is scope for improvement by offering a more explicit evaluation of the study's strengths and limitations, which would prove beneficial.

Revision: Thank you for your suggestion. In the revised version, we offered a more explicit evaluation of the study's limitations and strengths in the DISCUSSION section:

"However, this study has certain limitations. Firstly, in order to really demonstrate the effects on inflammation and IR in this study are just related to glucose per se and not to hyperosmolarity induced by 60 mmol/L glucose, we used L-glucose as an osmotic control for 60 mmol/L D-glucose. So, future researches maybe needed to analyze the damage of hyperosmolarity caused by HG to inflammation and insulin sensitivity. Further, considering the sample
size (n = 6 per group) may be relatively small in this study, more samples would be needed for further research".

"This is the first research about the mediation of macrophages to HG-related myogenic inhibition and IR in myoblasts, which supported macrophage may serve as a promising therapeutic target for skeletal muscle atrophy and IR. This study provides new insights into the prevention and treatment of skeletal muscle atrophy and IR, which may contribute to further explore in vivo the pathogenesis of IR and the involvement of macrophages".

Rond 2

Reviewer 1
Specific Comments to Authors:
It is a well-design study adding new information to the literature. According to my knowledge, it is a novel paper in its field opening new horizons for further evidence. Authors, succeed to present their findings in a clear way. In addition, the object as well as the results are appropriately discussed in the context of previous literature explaining the importance of the manuscript in its field. Authors succeed to present their data in a clear way adding information to the existing literature. Therefore, I have no corrections or further work to propose for the improvement of the manuscript and therefore it can be published unaltered. Best regards,

To follow, answers to the reviewer:

Dear reviewer,
Thank you for your comment.
Reviewer 2

Specific Comments to Authors:

First of all, please try to get at least two review reports before sending the manuscript to us (Editors) for making a decision. Since only one review report was received for this manuscript so I also had a closure look on this manuscript and made some corrections and comments. Please follow the following two things before sending the manuscript back to me again to make a final decision: (1) Please send the highlighted revised manuscript with the response to reviewers to original reviewer to get report from him/her whether all the concerns raised were addressed satisfactorily or not. I need the feedback from reviewer. (2) Please send the enclosed track-changed manuscript to the authors again to do the suggested corrections and reply to the comments I made on the manuscript. Once I received the further revised version of the manuscript and response from the original reviewer, I will make the final decision. Regards,

Prof. Islam

To follow, answers to the reviewer:

Dear reviewer,

Thank you sincerely for your meticulous reading of this manuscript and for the valuable suggestions and comments you've generously provided. The manuscript has greatly benefited from your insightful feedback. In the revised version, we have further refined the language in accordance with your recommendations. We have emailed Science Editor the revised manuscript and the point-by-point response. Thank you for your consideration. Best regards,

Ai

The author has revised the manuscript based on your comments, and replied point-to-point in the enclosed track-changed manuscript.