



PEER-REVIEW REPORT

Name of journal: *World Journal of Diabetes*

Manuscript NO: 72326

Title: Clopidogrel Delays and Can Even Reverse the Pathogenesis of Diabetic Nephropathy in Type 2 Diabetic db/db Mice, Likely through the Inhibition of Renal Inflammation

Provenance and peer review: Unsolicited Manuscript; Externally peer reviewed

Peer-review model: Single blind

Reviewer’s code: 05249683

Position: Editorial Board

Academic degree: BSc, MSc, PhD

Professional title: Professor

Reviewer’s Country/Territory: Egypt

Author’s Country/Territory: China

Manuscript submission date: 2021-10-18

Reviewer chosen by: AI Technique

Reviewer accepted review: 2021-10-25 09:04

Reviewer performed review: 2021-10-26 08:56

Review time: 23 Hours

Scientific quality	<input type="checkbox"/> Grade A: Excellent <input checked="" type="checkbox"/> Grade B: Very good <input type="checkbox"/> Grade C: Good <input type="checkbox"/> Grade D: Fair <input type="checkbox"/> Grade E: Do not publish
Language quality	<input type="checkbox"/> Grade A: Priority publishing <input checked="" type="checkbox"/> Grade B: Minor language polishing <input type="checkbox"/> Grade C: A great deal of language polishing <input type="checkbox"/> Grade D: Rejection
Conclusion	<input type="checkbox"/> Accept (High priority) <input type="checkbox"/> Accept (General priority) <input type="checkbox"/> Minor revision <input checked="" type="checkbox"/> Major revision <input type="checkbox"/> Rejection



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Re-review	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
Peer-reviewer statements	Peer-Review: <input checked="" type="checkbox"/> Anonymous <input type="checkbox"/> Onymous Conflicts-of-Interest: <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No

SPECIFIC COMMENTS TO AUTHORS

In this manuscript, the authors have a good finding in type 2 diabetic db/db mice. They found that clopidogrel reduced renal collagen deposition and fibrosis in db/db mice and prevented renal dysfunction. This occurs most likely by inhibiting renal macrophage infiltration and associated inflammation. Furthermore, the results are well-represented, and the units are clearly expressed. Also, the immunohistochemical staining results were achieved with a high level of expertise. On the other hand, the authors have to show the results of blood sugar and insulin assays in their manuscript to know the diabetic state in db/db mice. A complete information of db/db mice must be given in introduction and the written link in page 7 does not have any knowledge about these mice.



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Responses to the Editor and Reviewers

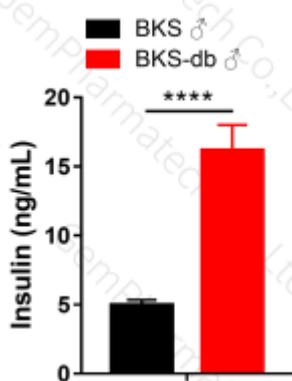
We thank the editor and all reviewers for the overall positive assessments on our manuscript. Accordingly, we have revised the manuscript as suggested. All the comments and suggestions have helped us to significantly improve our work and further increase the impact of this study. We have highlighted the changes in **red text** in the revised version of the manuscript (file labeled as **Red Copy**). Our point-to-point responses to the reviewers' questions and comments are provided below (all page numbers stated below refer to the **Red Copy** version of the manuscript **with Simple Markup** in the Review function). **Red Copy version was uploaded as "Supplementary Material" to the system. "72326_Auto_Edited" is the final version.**

Comment: On the other hand, the authors have to show the results of blood sugar and insulin assays in their manuscript to know the diabetic state in db/db mice. A complete information of db/db mice must be given in introduction and the written link in page 7 does not have any knowledge about these mice.

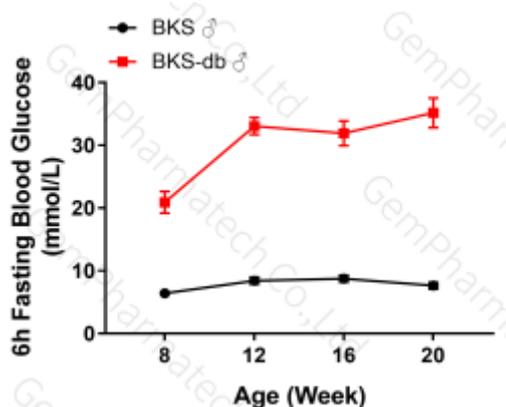
Response: Thank very much for the reviewer's insightful comments and suggestions. We have provided one sentence at the end of Introduction (page 4) and more detail description in at the beginning of Experimental animal of Material and Methods (page 4 - 5) to induce this mouse line with appropriate citation of a few new references.

As stated in the new description (page 4-5), the company has well and detail characterized these measurements for diabetic diagnosis in this mouse line, including "..... significant increases in body weight starting at 4 weeks, hyperglycemia (6-hour fasting blood glucose and HbA1c) at 8 weeks, insulinemia (>3-fold) at 8 week, and hyperlipidemia (cholesterol, triglyceride, and Low-density lipoprotein), along with the early onset of renal dysfunction, showed by significantly increased microalbumin level in 24-hour urine at 12 weeks of age.", see the link: <https://www.gempharmatech.com/shop/detail/3913.html>, we did immediately measure these blood glucose and insulin levels of these 8-week old mice once we noticed the obese db/db mice compared to their controls, which is consistent with what described by the Website of this company (see below figures from the Website). Due to our overwhelmingly trusting the company and also the expensive price for the db/db mice, we did not scarify any mice to measure blood insulin for these mice. However, when these mice have adapted the housing condition for 4 weeks until 12 weeks old, we have started dynamically examining body weight and their 6-hr fasting glucose levels of these db/db and WT mice, showing significantly higher fasting blood glucose level in db/db mice than the WT mice (Fig. 1). We also choose 12-week-old as the baseline, and started to administer clopidogrel at 12 weeks of age and forward at one of three doses (5, 10, or

20 mg/kg). Therefore, based on the baseline results (body weight, blood glucose level, Figure 1 and renal dysfunction, Figure 3), we did not have any doubt the model of this mouse as T2D. Hopefully what we thought and provided here are kind of acceptable.



Insulin levels of 8-week-old BKS-db mice are much higher than BKS mice (n=5). $P < 0.001$.



Blood glucose level after 6 hr fasting in BKS-db mice at the different ages (n=5-6).



PEER-REVIEW REPORT

Name of journal: *World Journal of Diabetes*

Manuscript NO: 72326

Title: Clopidogrel Delays and Can Even Reverse the Pathogenesis of Diabetic Nephropathy in Type 2 Diabetic db/db Mice, Likely through the Inhibition of Renal Inflammation

Provenance and peer review: Unsolicited Manuscript; Externally peer reviewed

Peer-review model: Single blind

Reviewer's code: 05947685

Position: Peer Reviewer

Academic degree: MD, PhD

Professional title: Doctor, Lecturer

Reviewer's Country/Territory: Thailand

Author's Country/Territory: China

Manuscript submission date: 2021-10-18

Reviewer chosen by: AI Technique

Reviewer accepted review: 2021-11-02 16:03

Reviewer performed review: 2021-11-10 05:12

Review time: 7 Days and 13 Hours

Scientific quality	<input type="checkbox"/> Grade A: Excellent <input type="checkbox"/> Grade B: Very good <input checked="" type="checkbox"/> Grade C: Good <input type="checkbox"/> Grade D: Fair <input type="checkbox"/> Grade E: Do not publish
Language quality	<input checked="" type="checkbox"/> Grade A: Priority publishing <input type="checkbox"/> Grade B: Minor language polishing <input type="checkbox"/> Grade C: A great deal of language polishing <input type="checkbox"/> Grade D: Rejection
Conclusion	<input type="checkbox"/> Accept (High priority) <input type="checkbox"/> Accept (General priority) <input type="checkbox"/> Minor revision <input checked="" type="checkbox"/> Major revision <input type="checkbox"/> Rejection
Re-review	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
Peer-reviewer statements	Peer-Review: <input checked="" type="checkbox"/> Anonymous <input type="checkbox"/> Onymous Conflicts-of-Interest: <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No



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SPECIFIC COMMENTS TO AUTHORS

In this manuscript, the authors showed that copidogrel can reduce the pathogenesis of diabetic nephropathy in db/db type 2 diabetic mice. The propose mechanism(s) are via the inhibition of inflammation as well as inhibiting the infiltration of macrophage to renal tissues. Even the research topic and findings are of interest, the results leading to the the authors' conclusion are still weak. Most of the findings are descriptive and may need more investigation to make a convincible and concrete conclusion. The authors may have to consider these following points: Major points: 1. The details of animal experiments need to be given and included in the Materials and Methods, rather than citing the previous publication (as detailed as other researchers could repeat the experiments after reading). The dosage, route, frequency of copidogrel administration mist be given. The graph comparing urinary albumin/creatinine ratio in Fig 3 should be clearly spelled out when the baseline was taken. The statement about the ethic approval with the number of approval should also be included in the section of animal experiments. 2. The authors suggested that copidogrel may inhibit the renal inflammation by demonstrating the reduction of macrophage infiltration and suppressing the expression of several genes, e.g., TNFA, IL1B. However, the supporting evidence to show how copidogrel exerts the activities on these local targets is unclear. There is neither supporting experiments nor the discussion on this point. On the other hand, copidogrel is known to directly affect the platelets. However, the systemic inflammatory cytokines which may be derived from platelets have not been examined yet in this manuscript. The levels of related cytokines in sera of the mice in each group will be strengthen the findings and conclusion of the authors. Minor points: 1. In page 7, there is a description that diabetic mice had longer bleeding time than in wild type, however, the results in Fig 2 does not show the concordance with the main text. 2. How did the authors prove the hypermutation of membranes. There is no experiment shown but mentioned in "Clopidogrel ameliorated diabetes-associated renal dysfunction, glomerular sclerosis, and collagen fiber deposition in the mice" section. 3. The scale bars should be added into every microscopic photos. 4. The IHC photos in Fig 4 are not clear, especially there is a very high background of Fig 4B. Could these figures be improved? 5. Please check the scale of graph in Fig 1B. The blood glucose levels are to low for the animal to be survive.



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Responses to the Editor and Reviewers

We thank the editor and all reviewers for the overall positive assessments on our manuscript. Accordingly, we have revised the manuscript as suggested. All the comments and suggestions have helped us to significantly improve our work and further increase the impact of this study. We have highlighted the changes in **red text** in the revised version of the manuscript (file labeled as **Red Copy**). Our point-to-point responses to the reviewers' questions and comments are provided below (all page numbers stated below refer to the **Red Copy** version of the manuscript **with Simple Markup** in the Review function). **Red Copy version was uploaded as "Supplementary Material" to the system. "72326_Auto_Edited" is the final version.**

Major points:

Comment 1. The details of animal experiments need to be given and included in the Materials and Methods, rather than citing the previous publication (as detailed as other researchers could repeat the experiments after reading). The dosage, route, frequency of copidogrel administration must be given. The graph comparing urinary albumin/creatinine ratio in Fig 3 should be clearly spelled out when the baseline was taken. The statement about the ethic approval with the number of approval should also be included in the section of animal experiments.

Response: The details of animal experiments have been described in Materials and methods (Pages 4 - 5), as responded to the above reviewer.

Now the detail dosage, route, frequency of copidogrel administration have been provided in the revised manuscript now on page 5. The graph comparing urinary albumin/creatinine ratio in Fig 3 has been clearly spelled out now (Figure 3A).

The statement of ethic approval has been given in this section (page 5) as suggested. The baseline has defined as 12 weeks old of these mice on page 5 too and in the legend of Fig 3.

Comment 2. The authors suggested that copidogrel may inhibit the renal inflammation by demonstrating the reduction of macrophage infiltration and suppressing the expression of several genes, e.g., TNFA, IL1B. However, the supporting evidence to show how copidogrel exerts the activities on these local targets is unclear. There is neither supporting experiments nor the discussion on this point. On the other hand, copidogrel is known to directly affect the platelets. However, the systemic inflammatory cytokines which may be derived from platelets have not been examined yet in this manuscript. The levels of related cytokines in sera of the mice in each group will be strengthen the findings and conclusion of the authors.



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Response: The inhibition of platelet aggregation and associated inflammatory markers release by Clopidogrel was reported in different pathological conditions such as coronary artery diseases, diabetes, and acute ischemic strokes. To confirm the systemic inflammation occurred in our diabetic model, we should examine the cytokines levels in serum and/or urine samples. However, since the work has been done more than a year ago, we had only focused on the renal function and pathologic changes, we did not well keep the serum samples. Therefore, what we can do is to measure the cytokine levels only in the urine samples of these mice in response to the reviewer's such suggestive and important issue. The ELISA results showed that diabetes caused significant increases in urine levels of these cytokines, clopidogrel dose-dependently reduced the levels of urinary TNF- α and MCP-1 in db/db mice, and only slightly increase in IL-6 levels, which remains supportive for our renal results and indirectly suggestive of the occurrence of systemic inflammation. Therefore, these results have been added as new Figure 6, with result description (page 10) in the revised manuscript.

Minor points:

Comments: 1. In page 7, there is a description that diabetic mice had longer bleeding time than in wild type, however, the results in Fig 2 does not show the concordance with the main text. 2. How did the authors prove the hypermutation of membranes. There is no experiment shown but mentioned in "Clopidogrel ameliorated diabetes-associated renal dysfunction, glomerular sclerosis, and collagen fiber deposition in the mice" section. 3. The scale bars should be added into every microscopic photos. 4. The IHC photos in Fig 4 are not clear, especially there is a very high background of Fig 4B. Could these figures be improved? 5. Please check the scale of graph in Fig 1B. The blood glucose levels are to low for the animal to be survive.

Response: Thank you so much for these excellent suggestions.

1. The description about the bleeding time was not accurate. We have revised it as "There was no difference between diabetic mice and WT non-diabetic mice in the baseline level, and treatment of the diabetic mice with clopidogrel increased the bleeding time in a dose-dependent manner." (page 8).
2. There maybe a mistake in the process of writing or editing, the "hypermutation of membranes" should be "hyperplasia of mesangial cells" (page 8).
3. We have added the scale bars into each photos of Figure 3, 4, 5, and 7.
4. For high background of collagen I, we examined it again, although it still has a background, but the difference between each group can be easily found (Figure 4 in revised manuscript).



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5. For figure 1B, the unit for blood glucose levels was wrong, it should be “mmol/L”, we have corrected it in the figure 1 now.



RE-REVIEW REPORT OF REVISED MANUSCRIPT

Name of journal: *World Journal of Diabetes*

Manuscript NO: 72326

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Reviewer's code: 05947685

Position: Peer Reviewer

Academic degree: MD, PhD

Professional title: Doctor, Lecturer

Reviewer's Country/Territory: Thailand

Author's Country/Territory: China

Manuscript submission date: 2021-10-18

Reviewer chosen by: Jing-Jie Wang (Online Science Editor)

Reviewer accepted review: 2022-02-04 12:21

Reviewer performed review: 2022-02-05 15:30

Review time: 1 Day and 3 Hours

Scientific quality	<input type="checkbox"/> Grade A: Excellent <input type="checkbox"/> Grade B: Very good <input checked="" type="checkbox"/> Grade C: Good <input type="checkbox"/> Grade D: Fair <input type="checkbox"/> Grade E: Do not publish
Language quality	<input checked="" type="checkbox"/> Grade A: Priority publishing <input type="checkbox"/> Grade B: Minor language polishing <input type="checkbox"/> Grade C: A great deal of language polishing <input type="checkbox"/> Grade D: Rejection
Conclusion	<input type="checkbox"/> Accept (High priority) <input checked="" type="checkbox"/> Accept (General priority) <input type="checkbox"/> Minor revision <input type="checkbox"/> Major revision <input type="checkbox"/> Rejection



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Peer-reviewer statements	Peer-Review: [<input checked="" type="checkbox"/>] Anonymous [<input type="checkbox"/>] Onymous Conflicts-of-Interest: [<input type="checkbox"/>] Yes [<input checked="" type="checkbox"/>] No
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SPECIFIC COMMENTS TO AUTHORS

The article has been improved. The answers addressed to this reviewer are acceptable and the limitation of the study is acknowledged.

Response:

We really appreciate your approval of our work. Thank you for your comments and suggestions which helped us to significantly improve our work.



RE-REVIEW REPORT OF REVISED MANUSCRIPT

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Conclusion	<input type="checkbox"/> Accept (High priority) <input type="checkbox"/> Accept (General priority) <input type="checkbox"/> Minor revision <input checked="" type="checkbox"/> Major revision <input type="checkbox"/> Rejection



Peer-reviewer statements	Peer-Review: [<input checked="" type="checkbox"/>] Anonymous [<input type="checkbox"/>] Onymous Conflicts-of-Interest: [<input type="checkbox"/>] Yes [<input checked="" type="checkbox"/>] No
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SPECIFIC COMMENTS TO AUTHORS

The authors responded to my concerns about blood glucose and insulin levels in the original version with a convincing response. **The response isn't in the revised version's or the supplementary file's file.**

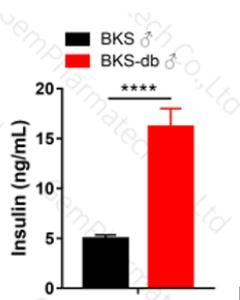
Response:

Thank very much for the reviewer's suggestions. We felt sorry that we might not clearly described well, which caused the reviewer's confusion a little bit.

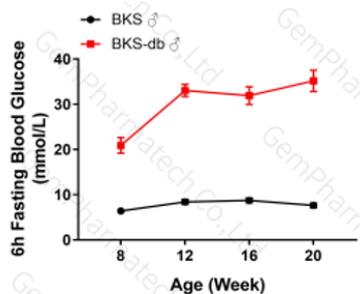
The suggestion by the reviewer for the first version is "..... On the other hand, the authors have to show the results of blood sugar and insulin assays in their manuscript to know the diabetic state in db/db mice. A complete information of db/db mice must be given in introduction and the written link in page 7 does not have any knowldege about these mice." This mouse line is commercially available and extensively used already as type 2 diabetic mouse line, instead of the new mouse line we made. In addition, the mouse is also very expensive; therefore, we purchased these well-recognized mouse line **without scarifying them immediately after purchased to double check whether their insulin levels and lipid profiles as well as fasting glucose level. In response to the previous suggestions in the first revision (R1), we explained this in detail, to express the description by the company and figures provided by the company (see below too) for the reviewer's reference in the response letter.**

*"We have provided one sentence at the end of Introduction (page 4) and more detail description in at the beginning of Experimental animal of Material and Methods (page 4-5) **to introduce this mouse line with appropriate citation of a few new references.** As stated in the new description (page 5), the company has well characterized these measurements in detail for diabetic diagnosis in this mouse line, including "..... significant increases in body weight starting at 4 weeks, hyperglycemia (6-hour fasting blood glucose and HbA1c) at 8 weeks, insulinemia (>3-fold) at 8 week, and hyperlipidemia (cholesterol, triglyceride, and Low-density lipoprotein), along with the early onset of renal dysfunction, showed by significantly increased microalbumin level in 24-hour urine at 12 weeks of age.", see the link: <https://www.gempharmatech.com/shop/detail/3913.html>. We did do immediately measure these blood glucose and insulin levels of these 8-week old mice once we noticed the obese db/db mice compared to their controls, which is consistent with what described by the Website of this company*

(see below figures from the Website). Due to our overwhelmingly trusting the company and also the expensive price for the db/db mice, we did not scarify any mice to measure blood insulin for these mice.



Insulin levels of 8-week-old BKS-db mice are much higher than BKS mice (n=5). $P < 0.001$.



Blood glucose level after 6 hr fasting in BKS-db mice at the different ages (n=5-6).

<https://www.gempharmatech.com/shop/detail/3913.html>

However, when these mice have adapted the housing condition for 4 weeks until 12 weeks old after we purchased, we have started dynamically examining body weight and their 6-hr fasting glucose levels of these db/db and WT mice, showing significantly higher fasting blood glucose level in db/db mice than the WT mice (Fig. 1 of manuscript). We also choose 12-week-old as the baseline, and started to administer clopidogrel at 12 weeks of age and forward at one of three doses (5, 10, or 20 mg/kg, Fig. 1 of the manuscript). Therefore, based on the baseline results (body weight, blood glucose level, Figure 1 and renal dysfunction, Fig. 3), we confirmed these mice as typical type 2 diabetic model. Hopefully what we thought and provided here are kind of acceptable.

We did not show, in our previous revised manuscript, the figures above cited from the company in the response letter to the reviewers, because we cannot use the company data. We have double checked the revised manuscript with properly addressing this issue again. Hopefully this time we have clearly addressed it. We have indicated these descriptions again in the re-revised (R2) manuscript **in font red** (end of page 4 and top of page 5).