Professor Lu Cai,

Editor

World Journal of Diabetes

Re: Ms. Ref. No.: 95252

Dear Editor,

We sincerely thank the reviewers for their time and effort in reviewing our manuscript, “Mitigating Diabetes-Related Complications: Empowering Metformin with Cholecalciferol and Taurine Supplementation in Type 2 Diabetic rats” submitted to the World Journal of Diabetes. We appreciate their valuable feedback and support, and we have carefully addressed their suggestions to improve our manuscript.

We have incorporated revisions to reflect the suggestions provided by the reviewers. Our point-by-point responses to the referees' questions are appended to this letter, highlighting the changes made to the manuscript in response to their critiques.

Once again, we extend our gratitude to you for facilitating the peer review process and to the reviewers for their thorough and constructive feedback. We hope that, upon further review, our revised manuscript will be deemed suitable for publication.

Yours sincerely,

Fadwa Ayman

Mohamed S. Attia
Reviewer #1, (Revision ID: 02623966):

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.

We thank Reviewer #1 for the positive feedback on our manuscript. We are pleased that the reviewer found our study well-designed, and clearly presented. We appreciate your recognition of its contribution to the literature and your recommendation for publication without changes. Your encouraging comments are greatly appreciated.
This study investigated the effects of supplementing metformin treatment with cholecalciferol and taurine in a rat model of streptozotocin-induced type 2 diabetes. The authors measured various metabolic, biochemical, histological and immunohistochemical parameters. They found that the combination of metformin, cholecalciferol and taurine was more effective than metformin alone in improving glycemic control, lipid profiles, liver function, oxidative stress markers, and pancreatic islet structure and function. The authors conclude that this combination therapy may help prevent diabetes complications by mitigating oxidative stress-induced tissue damage. Originality and Significance: The topic of preventing diabetes complications using combination therapies is clinically important given the increasing global burden of type 2 diabetes. While previous studies have looked at metformin plus either cholecalciferol or taurine, the novelty of this study is evaluating the triple combination. The results, if validated in humans, could lead to improved treatment approaches for diabetes. However, the manuscript does not sufficiently highlight what is already known on this topic and the key knowledge gaps this study aims to address.

We sincerely thank Reviewer #2 for the spending the time to thorough review of our manuscript and for providing valuable and constructive remarks that has extensively improved the quality of our paper.

1. The introduction should provide more context on the limitations of current therapies and prior evidence on cholecalciferol and taurine in diabetes.

We thank the reviewer for this comment. The introduction was rewritten to include the limitations of conventional treatments and evidence from earlier studies about the role of taurine and cholecalciferol in diabetes. Please see the Introduction section.

“Despite advances in DM research, insights into its etiology, effects, and progression, and developments in insulin and its analogs, optimal glycaemic control remains challenging. For patients with T2DM, lifestyle modifications like diet and exercise are important, yet they are challenging to follow consistently [1]. There are currently two existing primary treatment protocols for T2DM: injections of insulin or insulin-like peptides and oral administration of hypoglycemic agents. Although these therapies are essential for the treatment of type 2
diabetes mellitus (T2DM), they also bear adverse side effects \[2\]. For instance, several prescription drugs, such as metformin, sulfonylureas, GLP-1 receptor agonists, and SGLT2 inhibitors, are involved with gastrointestinal issues, risk of hypoglycemia, weight gain, and an increased susceptibility to infection \[3\]. Aside from this, these medications can become less effective over time as the disease progresses, necessitating the need for dose adjustments or combination therapy, which complicates treatment regimens, and in turn, leads to poor adherence. Additionally, metformin has been a standard treatment for T2DM due to its remarkable ability to lower plasma glucose levels \[4\]. Despite this, metformin suffers from the lowest compliance of all oral antidiabetic medications due to minor to severe adverse effects that impair patient compliance. While metformin controls diabetes and provides hepatoprotection, patients are still experiencing liver-related complications, necessitating a more effective therapeutic option \[5\].”

2. Specify the source/supplier of the streptozotocin

We thank the reviewer for the feedback. Based on the reviewer’s recommendation, we have written the source of STZ, which is Sigma-Aldrich Chemical Corporation (St Louis, MO, USA). Please see section 2. Materials.

3. Clarify the timing of the post-STZ glucose measurement used to confirm diabetes.

Thank the reviewer for bringing this important issue to our attention. Based on this comment, our timeline for the weekly monitoring of blood glucose for treatment and supplements has been clarified (For six weeks, we performed weekly monitoring).

4. Provide more details on the high-fat diet composition.

We thank the reviewer for highlighting this concern. According to the reviewer’s instructions, we have added the HFD composition, which contained an approximate kcal percentage of 58% fat, 25% protein, and 17% carbohydrates.

5. Specify whether the treatment allocations were randomized and if so, the randomization method.

We thank the reviewer for highlighting the concern about rat allocation. We have followed the simple randomization using an Excel sheet, through which we introduced all number of animals and then it was simply randomized. Please see the Method section.
6. Report the housing conditions (individual vs group) which can impact metabolic outcomes.

We thank the reviewer for highlighting the importance of mentioning the housing conditions. The housing condition was added to the Methods section as follows:

“Rats were obtained from the animal house at the Faculty of Veterinary Medicine of Zagazig University, where animals were kept in cages under standard conditions of temperatures around 20 to 25 °C with proper ventilation during light and dark cycles. Rats were left to acclimatize for one week before the experiment, during which they were fed with rat normal pellet diet and tap water.”

7. Specify the euthanasia method.

We appreciate the reviewer’s remark on euthanasia in our study. Based on your feedback, we have clarified the method we used for euthanasia, which was cervical dislocation. Please see tissue collection subsection in the Methods section.

8. Clarify the sample size per group for each outcome, as this is not always consistent in the results

Thanks to the reviewer for considering this important point in our manuscript. We have introduced the number per group for each outcome in each figure legend. The data generated from blood samples (biochemical analysis) were for six animals (n=6) from each group, while sample size (n = 4) from each group were used for immunohistochemical analysis and each animal study group was considered a single experimental unit.

9. However, the glucose data over time was analyzed by repeated measures of two-way ANOVA, which is the correct test, but this is not mentioned in the methods.

Thanks to the reviewer for raising this issue. In the Methods section, we specifically address and highlight the two-way ANOVA for the repeated measurements of blood glucose. Please see the statistical analysis section in Methods.

10. Please state the post-hoc test used for pairwise comparisons (Tukey's?)

Thanks to the reviewer for pointing out this concern. In the Methods section, we highlight this point under the statistical analysis subheading.
11. It would also be helpful to justify the sample size - was a power calculation done?

Thanks to the reviewer for this important remark. Mainly we have focused on the sample size calculation for the diabetic (test) groups (five groups) since the induction of diabetes has some risks of mortality. We provide a justification to calculate the sample size for an ANOVA using the following formula derived from Cohen’s power analysis for ANOVA:

\[ n = \left( \frac{(Z_{\alpha/2} + Z_{\beta})^2 \cdot (k - 1)}{f^2} \right) \]

Where:
- \( Z_{\alpha/2} \) is the critical value from the standard normal distribution for a significance level of \( \alpha \).
- \( Z_{\beta} \) is the critical value for power \((1 - \beta)\).
- \( k \) is the number of groups.
- \( f \) is the effect size.

Determine \( Z_{\alpha/2} \) and \( Z_{\beta} \):
- For \( \alpha = 0.05 \): \( Z_{0.025} \approx 1.96 \).
- For \( \beta = 0.20 \) (power of 0.80): \( Z_{0.20} \approx 0.84 \).
- Effect size (f): Suppose f=0.8.
- Number of groups (k): k=5.

Using these values in the formula:

\[ n = \left( \frac{(1.96 + 0.84)^2 \cdot (5 - 1)}{0.8^2} \right) \]

Simplifying:

\[ n = \left( \frac{(2.80)^2 \cdot 4}{0.64} \right) = \left( \frac{7.84 \cdot 4}{0.64} \right) = \left( \frac{31.36}{0.64} \right) = 49 \]

This suggests that a total sample size of approximately 50 is needed. This will be added to another ten rats of the nondiabetic (control group), so the total number of rats will be 60.

Our justification for the sample size was also confirmed by AI-guided power calculation after specifying the following parameters:

1- Effect Size (d): The magnitude of the difference we expect to detect between groups.
2- Significance Level (alpha): The probability of a Type I error (commonly set at 0.05).
3- Power (1 - beta): The probability of correctly rejecting the null hypothesis (commonly set at 0.80 or 80%).
4- Number of Groups: The number of independent groups in the study.
5- Sample Size per Group: The number of subjects in each group.

Given that we have five subgroups of 10 rats each, we'll perform the power calculation using these values.

Parameters for Calculation
1- Effect Size (d): 0.8 (assuming a moderate to large effect size, based on common practices in biological research)
2- Significance Level (alpha): 0.05
3- Power (1 - beta): 0.80
4- Number of Groups: 5
5- Sample Size per Group: 10

Power Calculation
We can use the statsmodels library in Python to perform this power calculation (AI-guided). Let's proceed with the calculation.

```python
import statsmodels.stats.power as smp

# Parameters
effect_size = 0.8  # Expected effect size (Cohen's d)
alpha = 0.05  # Significance level
power = 0.80  # Desired power
n_groups = 5  # Number of groups
sample_size_per_group = 10  # Sample size per group

# Calculate the total sample size
total_sample_size = sample_size_per_group * n_groups

# Perform power calculation
power_analysis = smp.FTestAnovaPower()
calculated_power = power_analysis.solve_power(effect_size=effect_size, nobs=total_sample_size, alpha=alpha, k_groups=n_groups, power=None)

calculated_power
```
The power calculation result shows that the study, with a total sample size of 50 rats (10 rats per group across 5 groups), has an approximate power of 0.996 (or 99.6%) to detect an effect size of 0.8 at a significance level of 0.05.

The sample size of 50 diabetic rats, with 10 rats per group, was justified based on a power calculation. The parameters for the calculation were:

- Effect Size (d): 0.8 (moderate to large effect size based on prior studies)
- Significance Level (alpha): 0.05
- Desired Power (1 - beta): 0.80 (80%)
- Number of Groups: 5

Using these parameters, the power analysis indicated a calculated power of 99.6%. This high power suggests that the sample size is more than adequate to detect the expected differences between the treatment groups, ensuring robust and reliable results.

Additionally, similar studies in the literature have used comparable sample sizes, further validating our chosen sample size for the study objectives.

12. The figures are informative, but the legends are very long - some of those details (scale bars, etc.) could be moved to the methods.

We appreciate the reviewer bringing attention to this issue of lengthy figure legends. Information about the scale bar has been moved to the Methods section under tissue collection and histological study.

13. Fig 1 - the asterisk and hash symbols are not defined in the legend.

Thanks to the reviewer for drawing our attention to this issue. Now Figure 1 has been modified to include the definition of these symbols. Please see Figure 1 legend.

14. Fig 2 - the y-axis units are missing for glucose and insulin.

Thanks for bringing this to our attention. Unfortunately, this mistake occurred when the image was copied directly from GraphPad Prism instead of exporting it. This mistake has been resolved by adding the image as it should.

15. Fig 4 - LDH does not seem to have been mentioned in the methods.
Thanks to the reviewer for pointing out that issue. We have highlighted the method for measurement of serum LDH activity, which was determined using the Spectrum kit according to Young’s (1997) procedure (42).

16. Fig 5 - the data suggest an increase in SOD and CAT with the combination treatment compared to control - this is not physiological and should be discussed.

Thanks to the reviewer for this very important remark. We have reviewed the reported mechanisms of Cholecalciferol and its relation to oxidative stress to validate our results. Numerous studies have documented the role of cholecalciferol in mitigating oxidative stress through diverse pathways. We have detailed the potential mechanisms relevant to our findings.

“A significant part of the protective action of CHO is to enhance the activities of key antioxidant enzymes, SOD and CAT (Figure 5). Also, CHO exerted a negative effect on MDA levels, which were in alignment with a meta-analysis of clinical data [6]. This can be explained by the fact that the CHO supplement interacts with Vitamin D receptors, which in turn activates the Vitamin D Response Elements (VDREs) in the nucleus, leading to the transcription of antioxidant genes [6]. Moreover, CHO also stimulates antioxidant pathways such as nuclear factor erythroid-2-related factor 2 (Nrf2)/Keap1[7] and ROS-scavenging enzymes (Glucose-6-phosphate dehydrogenase)[8] while reducing ROS-generating enzymes (NADPH oxidase) [9], thus indirectly reducing the risk of oxidative damage.”

17. Quantification of islet size/number and insulin staining intensity would strengthen the conclusions.

Thanks to the reviewer for this very important remark. We have considered this point and included the analysis of immunohistochemical results into our manuscript. These results have emphasized on the reversal of STZ action by Taurine and Cholecalciferol. We have added the methods for analysis which was conducted by Fiji software and introduced the results in Figure 9 and discussed the significance of these results. Please see the results and discussion sections.

Method:
“Photomicrographs at different magnifications 40X, and 400X. Fiji was used to analyze the stain intensity, number and size of the stained islets per 40x field. In order to evaluate stain intensity, images of test groups were first deconvoluted, then inverted, and then analyzed and compared with control measurements.”

Results

“In comparison with the control, the relative stain intensity was reduced by 32.7 and 24.3% in the STZ and MET groups, respectively (Figure 9a). Also, the number and size of islets were significantly lower than those of the control group (Figure 9b). Meanwhile, TAU exerted prominent restoration of insulin staining of the islets with restored and enhanced islets staining intensity with regular outlining comparable to control (Figure 9a). Also, the number of stained islets was increased to 5 stained area per field, whereas the area was increased to (7102 μm²). Eventually, rats supplemented with CHO and TAU experienced a significant rise in relative insulin stain intensity by 13.8% (Figure 9a), with greater stained islet size (8747 μm²) (Figure 9b).”

Discussion

“Immunohistochemical localization of insulin antibodies supported the microscopic H & E histopathological results of pancreas tissues. TAU treatment prominently restored insulin staining and islet size, with regular outlining comparable to the control. These findings were in line with those of Arany et al.18, who reported the increased pancreatic islet mass in TAU-supplemented rats, which could explain the augmented stain intensity for rats co-supplemented with CHO and TAU.”

18. There are quite a few typos and grammatical errors that need to be corrected.

Thanks to the reviewer for highlighting the grammatical mistakes. We have revised the manuscript language, and we sent the manuscript for Enago Service for English to avoid these mistakes.

19. The results should be put in the context of previous related studies on metformin, cholecalciferol, and taurine in animal models and humans.

Thanks to the reviewer for paying attention to the discussion section. We regret that our discussion section lacked connections to previous studies. Therefore, we have amended this
section to include the most relevant literature on the roles of Cholecalciferol and Taurine in both clinical and preclinical studies to strengthen our discussion. Could you please check the Discussion section?

20. **The potential mechanisms of the observed effects should be discussed in more depth.**

Thanks to the reviewer for bringing this concern to our attention. We regret that our study lacks a discussion about the mechanism of the observed effects. We have reintroduced our results in the discussion section in alignment with earlier studies on MET, TAU, and CHO and we supported that with references. Please see our rewritten Discussion section.

21. **The limitations of the animal model and ex vivo analyses for predicting human responses should be acknowledged.**

Thanks to the reviewer for highlighting this important and futuristic concern. Based on this suggestion, we have introduced the acknowledged limitations of animal models and their role in prediction in humans.

“Animal models offer limited predictive power.[10] This is attributed to the biological and physiological differences due to the discrepancy in how disease manifests and how treatment responds between rodents and humans. In contrast to animal models, the high genetic variation in drug metabolism and immune activity between individuals potentially affects estimates of treatment efficacy and toxicity.[11]”

22. **The potential side effects and contraindications of cholecalciferol and taurine in humans should be mentioned.**

Thank you for your valuable and constructive suggestion. We agree that it is important to mention the potential side effects and contraindications of both cholecalciferol and taurine in humans. We have now included this information in the revised manuscript. Please see the Introduction section.

For Taurine:

“Taking into account the negative aspects, numerous studies have been conducted on the effects of high TAU intake have not reported serious adverse effects (23–25). There could be
potential unwanted effects of TAU on the nervous system, cardiovascular system, and muscle emerging from its activity in regulating calcium release (26). Despite that TAU appears to be safe for humans at doses as high as 10 g/day for six months (25). However, as with any dietary supplement, TAU should be consumed moderately. When TAU is overconsumed, side effects may arise as nausea, vomiting, diarrhea, dizziness, tremors, and headache [2]. Furthermore, caution must be exercised due to the potential interactions between TAU and cardiovascular and nervous system drugs, which may exacerbate the effects of such treatments and increase the risk of hypotension [3], altered heart rhythms, or neurological concerns [4].”

For Cholecalciferol:

“Supplementation with cholecalciferol is unlikely to cause severe adverse reactions, even at high doses [12]. The excessive intake of this nutrient, on the other hand, may lead to hypercalcemia, that may cause calcium deposits in soft tissues, or lead to kidney stones, and cardiovascular complications [13,14]. Moreover, hypercalcemia can be exacerbated by CHO in individuals with high calcium levels, as well as hypertensive patients on thiazide diuretics [15]. In this regard, blood calcium levels are advised to be monitored to prevent CHO toxicity.”

23. Future directions should be proposed, such as dose-finding studies and clinical trials in patients.

Thanks to the reviewer for highlighting the importance of recommending future studies based on our research findings. We have outlined a series of studies that we propose should be conducted prior to clinical studies.

“Even so, it may still be necessary to conduct animal studies of TAU interaction prior to clinical studies to detect any potential pharmacokinetic interactions between the suggested combination therapy. Also, more parameters can be included in future glucose-insulin dynamic studies to assess insulin sensitivity either directly (hyperinsulinemic-euglycemic clamp) or indirectly (glucose tolerance tests). It will, therefore, be crucial to conduct dose-finding trials to identify effective and well-tolerated dosages of CHO and TAU to minimize metformin's adverse effects and optimize therapeutic outcomes in patients with diabetes. Furthermore, conducting clinical trials on diabetic patients can offer insight into the long-term benefits, safety profiles, and complications associated with the proposed co-therapy”.
Reviewer #3, (Revision ID: 03831562)

The authors would do well in future prospective studies to study the receptor and binding with reference to the ligand, 1,25 Dihydroxy cholecalciferol, as related to Type 2 diabetes mellitus

We appreciate Reviewer #3 for devoting time to read and evaluate the manuscript, as well as for providing constructive feedback on our paper.

2. Since Vitamin D levels have a role in insulin sensitivity, the authors can include more than one parameter to monitor insulin sensitivity in future studies

Thanks to the reviewer for emphasizing the importance of suggesting future studies based on our research findings. We have considered this suggestion and wrote about the insulin sensitivity studies in the proposed future studies.

“Even so, it may still be necessary to conduct animal studies of TAU interaction prior to clinical studies to detect any potential pharmacokinetic interactions between the suggested combination therapy. Also, more parameters can be included in future glucose-insulin dynamic studies to assess insulin sensitivity either directly (hyperinsulinemic-euglycemic clamp) or indirectly (glucose tolerance tests). It will, therefore, be crucial to conduct dose-finding trials to identify effective and well-tolerated dosages of CHO and TAU to minimize metformin's adverse effects and optimize therapeutic outcomes in patients with diabetes. Furthermore, conducting clinical trials on diabetic patients can offer insight into the long-term benefits, safety profiles, and complications associated with the proposed co-therapy.”