Author’s response to reviews

Title: High-dose clevudine impairs mitochondrial function and glucose-stimulated insulin secretion in INS-1E cells

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Author’s response to reviews: see over
Dear Editor

We are submitting the revised manuscript:

**Title:** High-dose clevudine impairs mitochondrial function and glucose-stimulated insulin secretion in INS-1E cells

**Author:** Yoon-Ok Jang, Xianglan Quan, Ranjan Das, Shanhua Xu, Choon-Hee Chung, Chan Mug Ahn, Soon-Koo Baik, In Deok Kong, Kyu-Sang Park and Moon Young Kim

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We are very pleased to have kind comments by reviewers. We tried to correct all points suggested by reviewers, which makes the manuscript much more precise and logical. We attached the ‘Answers to reviewers’ to the end of this letter.

We are looking forward to your decision, and thank you and reviewers for the time and effort in reviewing our manuscript.

Best regards,

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1st Reviewer’s Comments:

Major points:

1. Measurement of the activity of the cytochrome c oxidase on cell lysate...

To demonstrate the functional significance of decreased Cox1 mRNA, we performed the enzymatic activity measurement of cytochrome c oxidase (COX) based on the references provided by reviewer (Barrientos et al., 2002; Miro et al., 1998). We observed the reduced COX activity from clevudine (1mM)-treated cells (1027 ± 57 nmoles/min·mg protein) compared to that from control INS-1E cells (821 ± 70, n= 6, p<0.05). We added this data to the Results section (Figure 2B).

2. Measurement of oxygen consumption rate or citrate synthase activity ...

We agree that MTT assay is not enough specific to evaluate the mitochondrial activities. As reviewer’s suggestion, measurement of oxygen consumption rate or citrate synthase activity could provide more concrete evidence to prove the reduction in mitochondrial activity. We added this comments to the Discussion part.

3. The cause-effect relationship between the decreased ATP level and the impaired insulin release ...

The inhibitory effect of clevudine on insulin secretion was more sensitive than the effect on ATP level. We can speculate that the treatment of 100µM clevudine elicited significant reduction of ATP/ADP ratio which is the main signal for closing ATP-sensitive K+ channel and insulin exocytosis. In our INS-1E cells, we demonstrated that the glucose-stimulated insulin secretion (1.02 ± 0.15 % at 2.8mM; 3.93 ± 0.79 % at 16.7mM, n= 2) was completely abolished by the treatment with oligomycin (0.75 µg/ml), a mitochondrial ATP
synthase inhibitor (0.87 ± 0.08 % at 2.8mM glucose; 1.02 ± 0.03 % at 16.7mM, n= 2). As reviewer’s suggestion, this result demonstrates the cause-effect relationship between mitochondrial dysfunction and impaired insulin secretion. We included this finding to the Results section (Figure 5B).

**Minor points:**

4. **Clarify the reason of the long 4 weeks-treatment …**

We tested the effects of the duration for clevudine treatment on mtDNA depletion. Two weeks treatment with clevudine induced 39% reduction of mtDNA level (n=3), which was smaller than four weeks treatment (51%). The growth rate of INS-1E cells modestly slowed down by high-dose clevudine treatment, even though we did not perform the long-term estimation. However, there was no significant difference in protein amount between control cells (59 ± 3 µg; n=17) and cells incubated with 100µM (62 ± 4 µg; n=17) or 1mM clevudine (59 ± 3 µg; n=17) measured at 48 hours after seeding (2 x 10^5 cells) for functional assays.

5. **Normalization of mtDNA should be defined …**

We added the explanation about normalization of mtDNA or mRNA level in Materials and Methods section. We also added statistical significances in Figure 1 and 3C.

6. **MTT assay should be normalized to the cell number per well …**

We did not measure the cell number per well in the MTT assay. But as described above, we could not detect the difference in soluble protein amount between control and clevudine-treated cells during 48 hours incubation. We modified the description about cell number in the Results section to avoid the confusion or misunderstanding.
2nd Reviewer’s Comments:

Major points:

1. Statistical analysis is not appropriate...

In Figure 1, we presented relative mtDNA or mRNA levels of clevudine-treated cells, which are normalized to those of control cells. We performed a one-way ANOVA test, and modified p-values. For ATP and lactate data in Figure 3A & B, we compared the levels of ATP or lactate between control and clevudine-treated cells under low or high glucose condition, individually. We modified the graph and used Student’s t-test for that purpose.

2. PGC-1 alpha, Tfam, Nrf1 and SDH ...

We did quantitative RT-PCR for measuring transcriptional level of SDH in control and clevudine (100µM & 1mM)-treated cells. We also did further more experiments to quantify the mRNA level of PGC-1alpha, Tfam, and Nrf1. We made a new graph about the transcriptional changes of these genes upon clevudine treatment and included in the Figure 1C.

3. Impairment of fatty acid oxidation leading to lipid accumulation...

As reviewer’s appropriate suggestion, we performed Oil-red O staining to control and clevudine-treated cells. Without exogenous fatty acid loading, there was no significant difference between two groups. When we incubated cells with a mono-unsaturated fatty acid, 0.7mM oleate, clevudine-treated
cells showed a pronounced lipid accumulation, which was much less in control cells. We included this result as Figure 4.

4. Possibility that clevudine could impair mitochondrial function through mechanism other than mtDNA depletion...

We really appreciate very helpful comments by reviewer. We included this explanation to the Discussion section.

**Minor points:**

1. *More pieces information should be provided*...

   We added explanation about cell line in the Materials and Methods section.

2. *Should specify about 4 weeks of clevudine treatment* ...

   We add this description in the Materials and Methods section as well as Results section.
3. Uridine is able to prevent NRTI-induced mitochondrial dysfunction …

We would like to express a gratitude for reviewer’s comments. We added the description about uridine as a candidate for therapeutic application in the Discussion part.