

RESEARCH ARTICLE

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

Yoon-Ok Jang<sup>1</sup>, Xianglan Quan<sup>2</sup>, Ranjan Das<sup>2</sup>, Shanhua Xu<sup>2</sup>, Choon-Hee Chung<sup>1,4</sup>, Chan Mug Ahn<sup>3,4</sup>, Soon-Koo Baik<sup>1,4</sup>, In Deok Kong<sup>2,4</sup>, Kyu-Sang Park<sup>2,4\*</sup> and Moon Young Kim<sup>1,4\*</sup>

## Abstract

**Background:** Clevudine is a nucleoside analog reverse transcriptase inhibitor that exhibits potent antiviral activity against hepatitis B virus (HBV) without serious side effects. However, mitochondrial myopathy has been observed in patients with chronic HBV infection taking clevudine. Moreover, the development of diabetes was recently reported in patients receiving long-term treatment with clevudine. In this study, we investigated the effects of clevudine on mitochondrial function and insulin release in a rat clonal  $\beta$ -cell line, INS-1E.

**Methods:** The mitochondrial DNA (mtDNA) copy number and the mRNA levels were measured by using quantitative PCR. MTT analysis, ATP/lactate measurements, and insulin assay were performed.

**Results:** Both INS-1E cells and HepG2 cells, which originated from human hepatoma, showed dose-dependent decreases in mtDNA copy number and cytochrome c oxidase-1 (Cox-1) mRNA level following culture with clevudine (10  $\mu$ M-1 mM) for 4 weeks. INS-1E cells treated with clevudine had reduced total mitochondrial activities, lower cytosolic ATP contents, enhanced lactate production, and more lipid accumulation. Insulin release in response to glucose application was markedly decreased in clevudine-treated INS-1E cells, which might be a consequence of mitochondrial dysfunction.

**Conclusions:** Our data suggest that high-dose treatment with clevudine induces mitochondrial defects associated with mtDNA depletion and impairs glucose-stimulated insulin secretion in insulin-releasing cells. These findings partly explain the development of diabetes in patients receiving clevudine who might have a high susceptibility to mitochondrial toxicity.

**Keywords:** clevudine, mitochondrial DNA, mitochondrial dysfunction, glucose-stimulated insulin secretion

## Background

Chronic infection with hepatitis B virus (HBV) frequently leads to serious liver disease such as cirrhosis, fulminant hepatic failure, and hepatocellular carcinoma [1]. Several antiviral drugs have been developed and prescribed for HBV infection. Commonly used antiviral therapies are nucleoside analog reverse transcriptase inhibitors (NRTIs) including entecavir, lamivudine, and telbivudine. NRTIs undergo intracellular and

intramitochondrial phosphorylation into active triphosphates that are capable of inhibiting HIV reverse transcriptase (RT) [2]. However, these drugs have side effects such as lipodystrophy, neuropathy, myopathy, and liver steatosis, all of which are related to mitochondrial toxicity. *In vitro* and *in vivo* studies have shown that some NRTIs inhibit DNA polymerase- $\gamma$ , a nuclear-encoded polymerase important for mitochondrial DNA (mtDNA) replication [3,4]. Depletion of mtDNA induced by NRTIs may attenuate mitochondrial oxidative phosphorylation, which could limit their clinical use.

Clevudine (1-(2-deoxy-2-fluoro- $\beta$ -L-arabinofuranosyl)thymine) is an NRTI that exhibits potent and sustained

\* Correspondence: qsang@yonsei.ac.kr; drkimmy@yonsei.ac.kr

<sup>1</sup>Department of Internal Medicine, Yonsei University, Wonju College of Medicine, Wonju, 220-701, Korea

<sup>2</sup>Department of Physiology, Yonsei University, Wonju College of Medicine, Wonju, 220-701, Korea

Full list of author information is available at the end of the article

antiviral activity against HBV with weaker effects on mitochondrial structure and function compared to those of other NRTIs [2,5]. However, long-term therapy for more than one year results in the development of considerable drug resistance and skeletal myopathy [6-9]. Muscle biopsies from patients with myopathy as a complication of clevudine treatment revealed severe necrosis with cytochrome c oxidase (COX)-negative ragged red fibers, the typical phenotype of mitochondrial myopathy [7,10]. Clevudine-induced myopathy developed in approximately 4-5% of patients and was usually reversible after discontinuation of clevudine [9].

It is well known that mitochondria play a critical role in nutrient-stimulated insulin secretion, as well as in insulin actions at target cells [11]. Recently, a patient who developed diabetes mellitus after clevudine treatment was reported [12]. We hypothesized that the mitochondrial dysfunction invoked by clevudine treatment could be a precipitating factor in diabetogenesis. Until now, the majority of *in vitro* studies for antiviral agent toxicities have been performed in different cell types, yielding conflicting results [13-15]. Insulin-secreting cells are highly specialized fuel sensors that maintain blood glucose level in the body by monitoring the ATP/ADP ratio, which is strictly regulated by mitochondrial oxidative phosphorylation. Thus, insulin-secreting cells are an appropriate model system for identification of mitochondrial toxicity and its functional consequences following antiviral therapy. In this study, we investigated the effects of clevudine exposure on mtDNA content, mitochondrial function, and metabolism-secretion coupling in insulin-releasing cells to elucidate the mechanism underlying the reversible diabetes observed in clevudine-treated patients.

## Methods

### Cell culture and drugs

Clevudine was purified from Revovir<sup>®</sup> tablets (Bukwang Pharm. Co., Seoul, Korea). The amount of harvested clevudine was analyzed using HPLC (Agilent G1315B UV Diode array detector, AD, Santa Clara, CA, USA). A single peak with the expected amount of clevudine was measured based on the known weight of one tablet. INS-1E cell, a clonal pancreatic  $\beta$ -cell line received from Prof. Claes B. Wollheim, were cultured in complete medium composed of RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 50  $\mu$ M 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. HepG2 cells, a human hepatoma cell line, were grown in DMEM medium (Invitrogen) containing 5.6 mM glucose, 4 mM L-glutamine and 1 mM sodium pyruvate. For the following

experiments, cells were cultured with or without clevudine for 4 weeks.

### Quantitative PCR

Total DNA or RNA was isolated and purified from INS-1E and HepG2 cells using DNeasy or RNeasy kits (Qiagen, Valencia, CA, USA), respectively. To obtain cDNA, reverse transcription (RT) was performed with oligo-dT (Applied Biosystems, Foster City, CA, USA) using reverse transcriptase (Promega, Madison, WI, USA). For PCR amplification, sequence-specific oligonucleotide primers for the genes of interest were designed (Bioneer, Daejeon, Korea) based on rat and human sequences in the GenBank database (Table 1). Quantitative real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems) was performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol. All amplifications were followed by melting curve analysis. The  $\beta$ -actin was used as the reference gene, and relative abundance of DNA or mRNA in clevudine-treated cells was normalized to that level in control cells calculated by using  $2^{-\Delta\Delta C_t}$  method.

### MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). INS-1E cells seeded onto a 96-well plate

**Table 1 Primers for quantitative PCR**

Name		Primer sequence	Accession code	product size
rat $\beta$ -actin	+	ATGGTGGGTATGGGTCAGAA	NM_031144.2	100 bp
	-	TCCATATCGTCCCAGTTGGT		
rat Cox1	+	GGAGCAGTATTCGCCATCAT	NC_001665	90 bp
	-	GTGGGCTTTTGCTCATGTGT		
rat PGC1a	+	GGCACATCTGTTCTTCCACA	NM_031347.1	110 bp
	-	TTCTGGTCTTGGAGCTGTT		
rat NRF1	+	GGACAGCAAGCCATTGTTCT	NM_001100708.1	98 bp
	-	TACTTGCGCACCCACATTCTC		
rat Tfam	+	GCTGAGTGAAGGTGTACAAG	NM_031326.1	85 bp
	-	CTTCCTTCTAAGCCCATCAG		
rat SDH	+	TCTTCTACCCGCTCACAT	NM_130428.1	90 bp
	-	AATGCCATCTCCAGTTGTCC		
human $\beta$ -actin	+	AAGTTCACAATGTGGCCGAG	NM_001101.3	98 bp
	-	ATGGCAAGGGACTTCTGTGA		
human Cox1	+	CACACTCCACGGAAGCAATA	NC_012920.1	82 bp
	-	GCCACCTACGGTGAAAAGAA		

( $5 \times 10^4$  cells/well) were incubated with MTT (50  $\mu$ g/well) for 2 hrs, and then the medium was discarded and replaced with dimethylsulfoxide (100  $\mu$ l/well). The absorbance of each well was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader, after background subtraction at 650 nm.

#### Cytochrome c oxidase (COX) activity measurement

INS-1E cells were harvested and incubated with isosmotic medium [16] containing 0.2% triton X-100 at 30°C for 2 min. Enzymatic activity of COX was measured spectrophotometrically at 550 nm based on previous reports [16,17].

#### ATP and lactate measurements

INS-1E cells seeded onto 24-well plates ( $3 \times 10^5$  cells/well) were preincubated with glucose-free medium for 2 hrs prior to incubation with KRBH solution (135 mM NaCl, 3.6 mM KCl, 2 mM NaHCO<sub>3</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, 1.5 mM CaCl<sub>2</sub>, 10 mM HEPES, pH 7.4) containing 2.8 mM glucose for 30 min. The cells were then stimulated for 15 min with KRBH buffer at a low (2.8 mM) or high (16.7 mM) glucose concentration. The ATP content in the cell lysate (Roche HS-II Bioluminescence kit, Mannheim, Germany) and the lactate level in the cell supernatant (Biovision #K607-100, Mountain View, CA, USA) were measured as described previously [18]. Measurement of the protein concentration in cell lysates was performed using the Bradford assay.

#### Oil red staining

INS-1E cells on coverslip were treated with bovine serum albumin (BSA) or oleate, a monounsaturated fatty acid for 24 hours. After fixation with 10% formalin, cells were washed with 60% isopropanol and dried at room temperature. Cells were incubated with Oil Red O (Sigma, St. Louis, MO, USA) for 10 min, and then counterstained with hematoxylin.

#### Insulin measurement

INS-1E cells were seeded and cultured as for ATP and lactate measurement. For insulin measurement, 0.1% BSA was included in the KRBH solution, and the cells were stimulated with low or high concentrations of glucose for 30 min, as described previously [18]. Insulin levels in supernatant and cell extracts were measured using a rat insulin enzyme immunoassay kit (Shibayagi Co., Gunma, Japan).

#### Data analysis

All data are presented as mean  $\pm$  SEM, and the statistical significance was determined using One-way ANOVA or Student's t test.

## Results

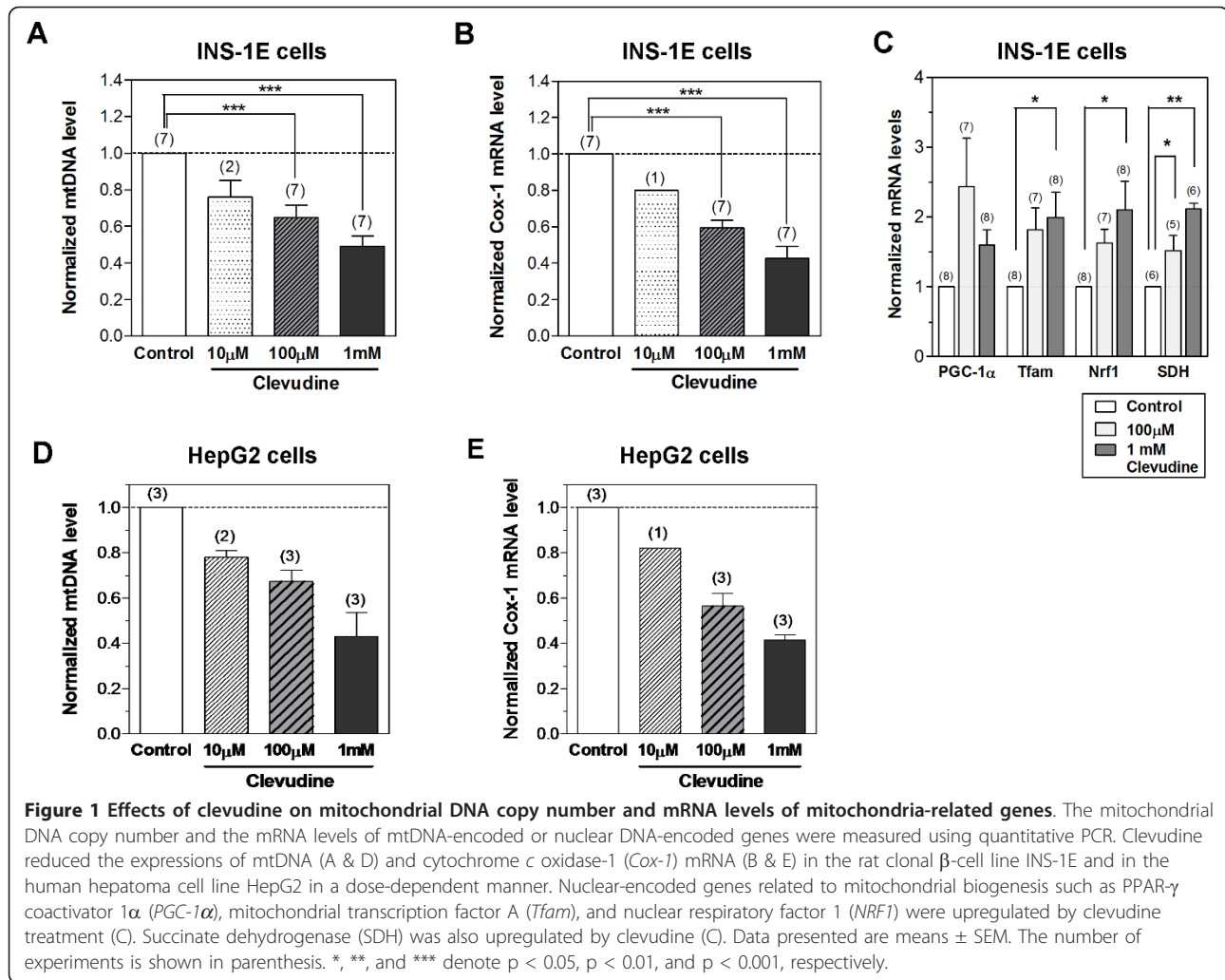
### Effects of clevudine on mtDNA copy number and mRNA levels of mtDNA encoded genes

INS-1E cells were cultured with different concentrations of clevudine for 4 weeks and the *in vitro* effects on mtDNA replication and translation were measured. Treatment with clevudine (10  $\mu$ M to 1 mM) reduced the mtDNA copy number in a dose-dependent manner (Figure 1A). Two weeks treatment with clevudine induced 39% reduction of mtDNA level ( $n = 3$ ), which was smaller than four weeks treatment (51%). The mRNA levels of mtDNA-encoded *Cox-1* were also dose-dependently attenuated by clevudine (Figure 1B). Interestingly, we observed upregulation of PPAR- $\gamma$  coactivator 1 $\alpha$  (*PGC-1 $\alpha$* ), mitochondrial transcription factor A (*Tfam*), and nuclear respiratory factor 1 (*NRF1*) in clevudine-treated INS-1E cells. Upregulation of these transcription factors could be a nuclear response to mitochondrial dysfunction [3]. Succinate dehydrogenase (SDH), a nuclear-encoded mitochondrial enzyme, was also upregulated by clevudine-treatment (Figure 1C). We next examined the effects of clevudine on the levels of mtDNA and RNA in the human hepatoma cell line HepG2, major target cells of insulin action. Clevudine showed suppressive effects on mtDNA replication and transcription in HepG2 cells, similar to the effect in INS-1E cells (Figures 1D and 1E).

### Mitochondrial dysfunction induced by clevudine

The amount of formazan reaction product formed in the MTT assay reflects the total mitochondrial enzymatic activity in each well. INS-1E cells treated with or without clevudine for 4 weeks were seeded 48 hrs before the MTT assay. Exposure to clevudine decreased the MTT absorbance (71% by 100  $\mu$ M and 56% by 1 mM, Figure 2A). However, we observed that there was no significant difference in protein amount between control cells ( $59 \pm 3$   $\mu$ g;  $n = 17$ ) and cells incubated with 100  $\mu$ M ( $62 \pm 4$   $\mu$ g;  $n = 17$ ) or 1 mM clevudine ( $59 \pm 3$   $\mu$ g;  $n = 17$ ) for 48 hours after seeding ( $3 \times 10^5$  cells). This result implies that reduction of MTT signal by clevudine might be resulted from decreased mitochondrial reducing capacity. To demonstrate the functional significance of decreased *Cox1* mRNA, we performed the enzymatic activity measurement of cytochrome c oxidase (Cox) based on previous reports [16,17]. We observed the reduced Cox activity of clevudine (1 mM)-treated cells compared to that of control INS-1E cells (Figure 2B).

We measured the cellular contents of ATP in control and clevudine-treated INS-1E cells using a bioluminescence method after incubation with low (2.8 mM) or high (16.7 mM) concentrations of glucose for 15 min. As shown in Figure 3A, cells cultured with 1 mM clevudine had lower cytosolic ATP level in both low and high glucose



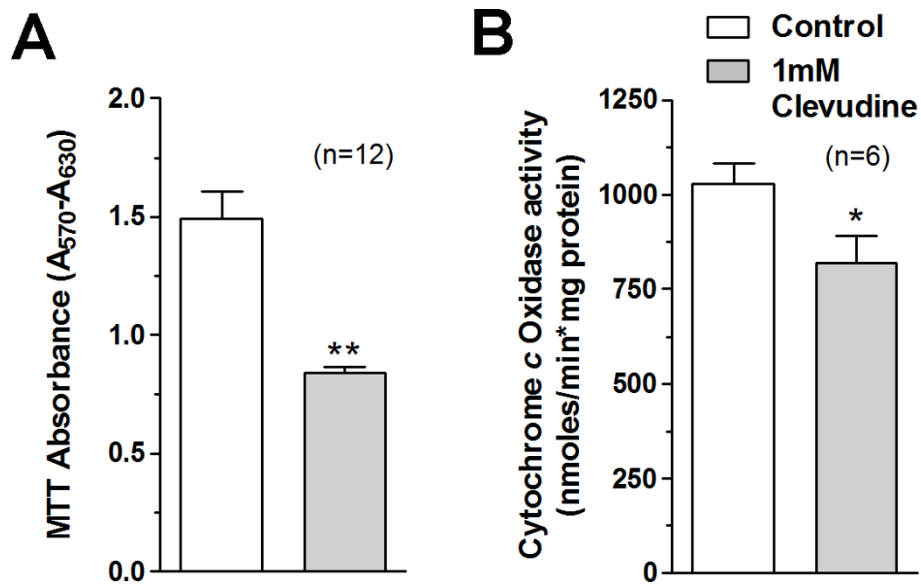
conditions than control cells. Lactate production from INS-1E cells was markedly elevated by incubation with glucose for 15 min (Figure 3B). The glucose-induced lactate production was increased in cells treated with 1 mM clevidine compared to that in control cells (Figure 3B).

One of the metabolic consequences of mitochondrial dysfunction is an impairment of fatty acid oxidation, thus leading to lipid accumulation [19,20]. To detect the lipid droplet in cytosol, we performed Oil-red O staining to control and clevidine-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, 0.7 mM oleate, clevidine-treated cells showed a pronounced lipid accumulation, which was much less in control cells (Figure 4).

#### Inhibition of glucose-stimulated insulin secretion by clevidine

To identify whether clevidine-induced mitochondrial dysfunction affects insulin secretory activity, we

measured the released and cellular contents of insulin via an enzyme immunoassay. The cellular insulin contents were not significantly different between control INS-1E cells ( $816 \pm 167$  ng/well) and cells treated with clevidine (1 mM) for 4 weeks ( $769 \pm 170$  ng/well). After incubation for 30 min with low (2.8 mM) or high (16.7 mM) concentrations of glucose, the released insulin was normalized to the cellular content and expressed as a percentage of the content released. We observed that high concentration glucose stimulated the release of insulin by 5.1-fold in control cells but by only 3.1-fold and 1.9-fold in cells treated with 100 μM and 1 mM clevidine for 4 weeks, respectively (Figure 3). There was no difference in % insulin releases induced by low concentration glucose between the control and clevidine-treated groups (Figure 5A). We observed that the glucose-stimulated insulin secretion was completely abolished by the treatment with oligomycin (0.75 μg/ml), a mitochondrial ATP synthase inhibitor (Figure 5B). This result demonstrates the cause-effect relationship



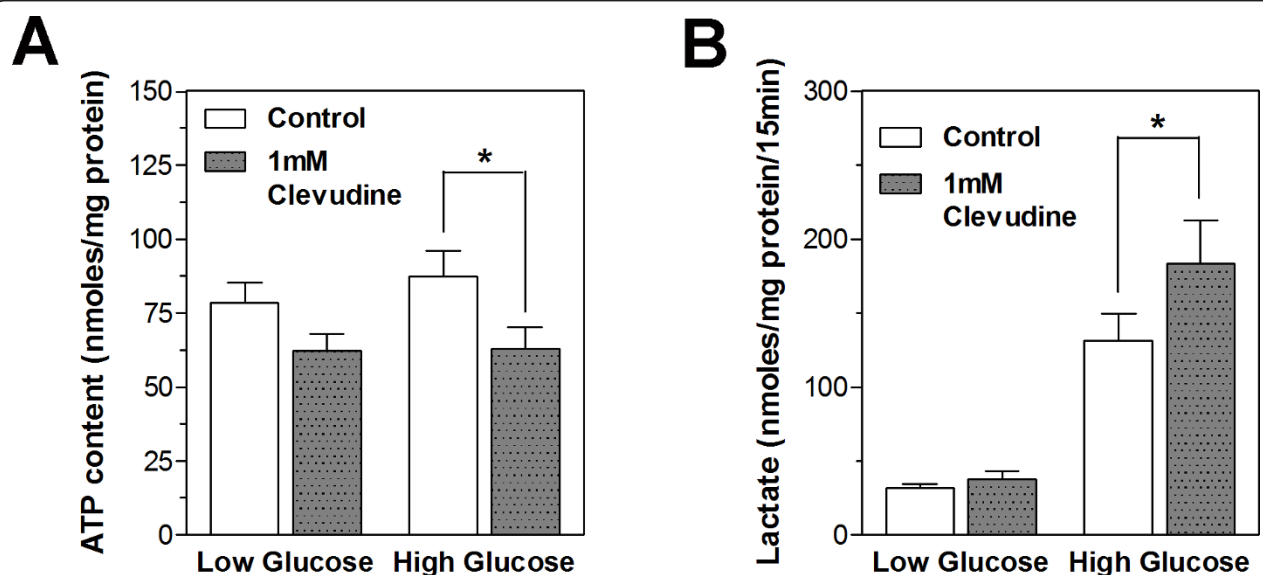
**Figure 2** High-dose clevudine decreased the mitochondrial activities in INS-1E cells. (A) Clevudine reduced the MTT signal, which reflects total mitochondrial activity within each well. (B) Clevudine decreased the enzymatic activity of cytochrome c oxidase (COX). Data are presented as means  $\pm$  SEM. The number of experiments is shown in parenthesis. \* and \*\* denote  $p < 0.05$  and  $p < 0.01$ , respectively.

between mitochondrial dysfunction and impaired insulin secretion.

### Discussion

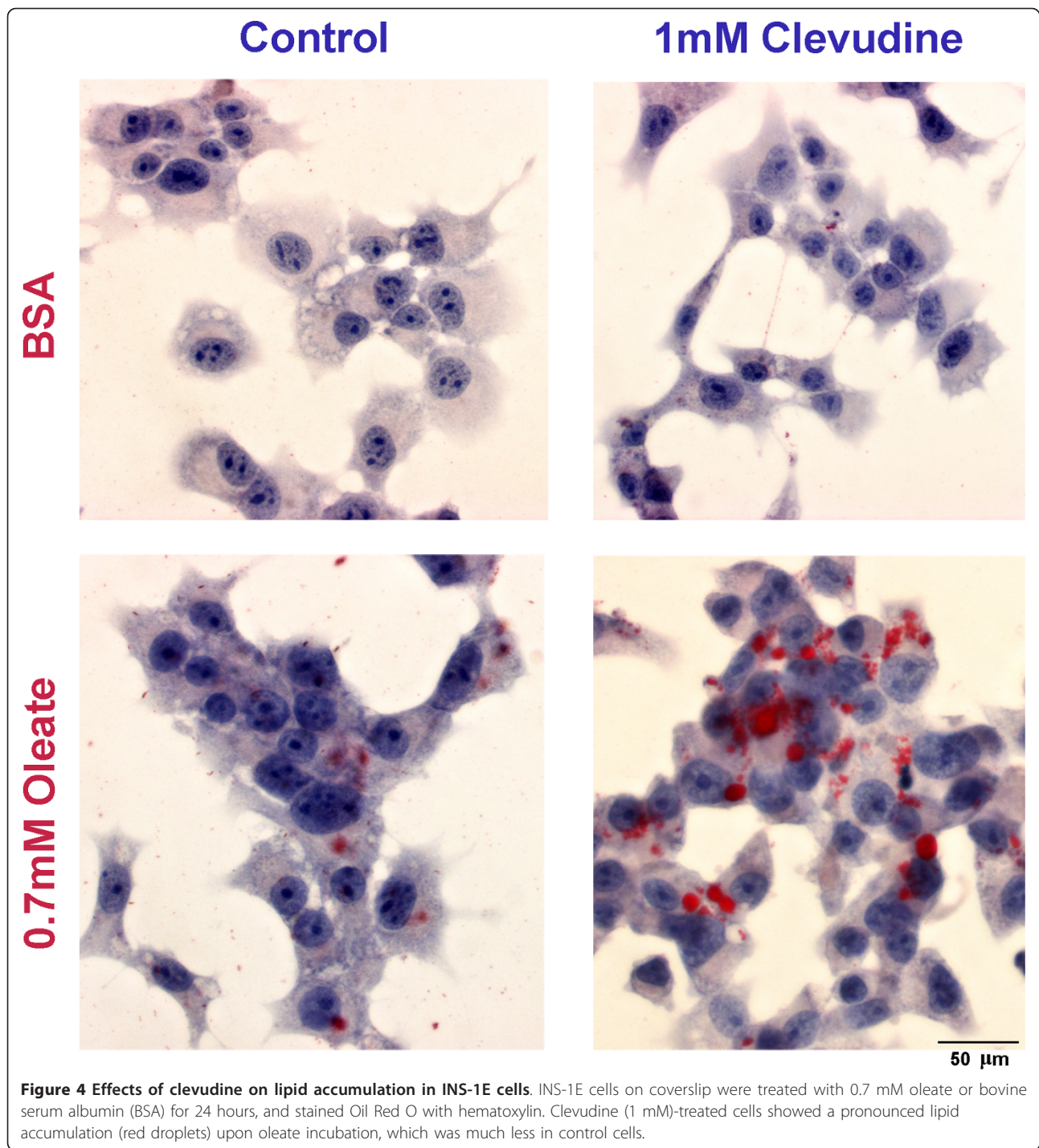
In pancreatic  $\beta$ -cells, mitochondria are of particular importance in the regulation of insulin secretion because they produce ATP as well as other coupling

factors which link nutrient metabolism and insulin exocytosis [11]. mtDNA-depleted  $\beta$ -cell lines show complete absence of nutrient-stimulated insulin secretion [21]. Patients with mtDNA mutations develop diabetes, accounting for up to 1% of the total number of diabetic patients [22]. Moreover, postmortem islets from type 2 diabetes patients display functional deterioration of



**Figure 3** High-dose clevudine decreased ATP level and increased lactate production in INS-1E cells. Cytosolic ATP level was reduced (A), but glucose-stimulated lactate production was enhanced (B) in 1 mM clevudine-treated cells. Data are presented as means  $\pm$  SEM. \* denotes  $p < 0.05$ .

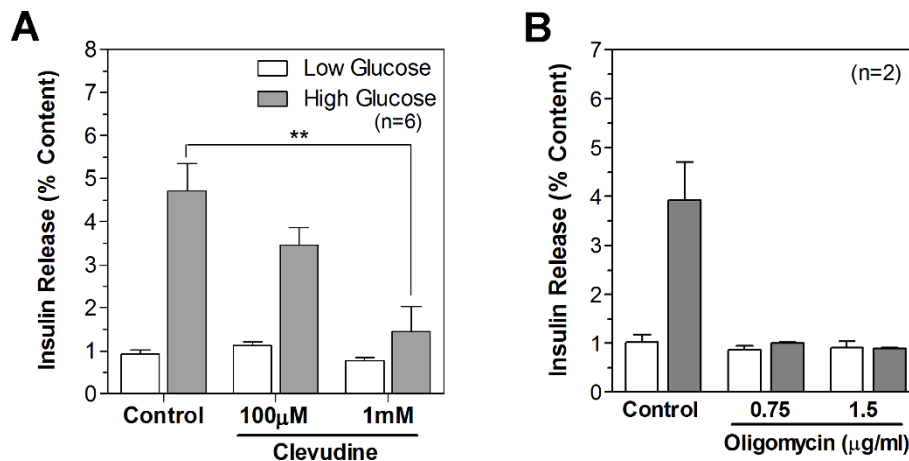




mitochondria [23]. Therefore, factors that disturb the mitochondrial function in pancreatic  $\beta$ -cells might affect metabolism-secretion coupling and diabetogenesis.

The present study showed that the effective anti-HBV agent clevedine has a negative effect on the copy number and transcription of mtDNA in insulin-releasing cells and hepatoma cells. The reduced expressions of

mtDNA-encoded proteins lead to attenuation of mitochondrial function. In insulin-releasing cells, clevedine-induced mitochondrial dysfunction can elicit defective insulin secretion in response to substrates for mitochondrial metabolism. To our knowledge, this is the first demonstration that an antiviral agent can impair nutrient-stimulated insulin secretion as a result of



**Figure 5 Clevedine inhibited glucose-stimulated insulin secretion.** Released insulin and cellular insulin content were measured using an enzymatic immunoassay after incubation with Krebs buffer containing 2.8 mM or 16.7 mM glucose for 15 min. Glucose-stimulated insulin secretion was impaired by clevedine incubation (A), as well as by inhibiting mitochondrial ATP synthase with oligomycin (B). Insulin release was normalized to the insulin content. Data presented are mean  $\pm$  SEM. \*\* denotes  $p < 0.01$ .

mitochondrial dysfunction. Because of their high dependency on mitochondrial function in metabolism-secretion coupling, insulin-secreting cells provide a useful model to investigate the functional consequences of drug-induced mitochondrial toxicity.

NRTIs are widely used to treat various viral diseases such as acquired immunodeficiency syndrome (AIDS) and hepatitis B [24]. However, *in vitro* studies showed that NRTIs can alter mtDNA content by inhibiting DNA polymerase- $\gamma$  [25]. Moreover, myopathy accompanied by mtDNA depletion has been reported in NRTI-treated patients [4]. Clevedine treatment has also been associated with the development of mitochondrial complications. In contrast to early studies [2], depletion of mtDNA in skeletal muscle has been observed in patients treated with clevedine [7,26]. Typical histological features of mitochondrial myopathy and abnormal mitochondrial morphology were displayed in tissues from patients with increased lactate dehydrogenase and lactate levels [8,10]. Although the incidence of clevedine-induced myopathy was reported to be low (~5%) [9], a substantial proportion (~14.5%) of clevedine-treated patients have been found to experience symptoms, signs, and laboratory abnormalities relevant to clevedine-induced myopathy [27].

To directly confirm the effects of clevedine on mitochondrial function, we cultured cells with medium containing different concentrations of clevedine for 4 weeks. Clevedine markedly decreased the MTT signal without significant changes in cellular protein implying the diminished enzyme activities for reduction of MTT. Since MTT assay is not specific to evaluate mitochondrial function, measurement of oxygen consumption

rate or citrate synthase activity could provide more concrete evidence to prove the mitochondrial defects. Consistent with mtDNA depletion, COX activity and cellular ATP content were reduced. Decreased mitochondrial fatty acid oxidation could induce triglyceride accumulation [19]. To avoid lipotoxic effects of palmitate in insulin-secreting cells [28], we loaded unsaturated fatty acid oleate for 24 hours, which elicited a marked increase of lipid accretion within clevedine-treated cells. The inhibitory effect of clevedine on insulin secretion was more sensitive than the effect on ATP level. We can speculate that the treatment of 100  $\mu$ M clevedine elicited significant reduction of ATP/ADP ratio which is the main signal for closing ATP-sensitive  $K^+$  channel and insulin exocytosis.

We also observed some compensatory responses to reduced mtDNA copy number and its functional consequences. First, PGC-1 $\alpha$  and its downstream transcriptional factors, NRF-1 and Tfam, were upregulated by clevedine. Second, nuclear DNA-encoded succinate dehydrogenase was also upregulated, which has already been observed in muscle of patients suffering from clevedine-induced myopathy [10]. Third, lactate production was modestly increased in association with diminished ATP content. Pancreatic  $\beta$ -cells and clonal  $\beta$ -cell lines are known to have very low lactate dehydrogenase levels, which contribute to their dependency on mitochondrial function. The increase in lactate production observed in our study also demonstrates that clevedine imposes selective defects on mitochondria rather than overall cytotoxicity.

In our study, mtDNA copy number in clevedine (1 mM)-treated cells was decreased to half of that in

control. It has been reported, however, that to evoke mitochondrial dysfunction mtDNA level should fall below 60% which was named as 'phenotypic threshold' [29]. This can be explained by genetic and functional complementation at the levels of transcription, translation, enzyme activity and cell activity. Several investigators showed that NRTI such as zidovudine and stavudine can also induce mitochondrial dysfunction independent from lack of mtDNA [20,30]. Thus, we cannot exclude the possibility that clevidine could be involved in multiple site of inhibition of mitochondrial function in addition to the effects of mtDNA depletion.

Niu et al. [31] suggested that the intracellular level of the triphosphate form of clevidine in cells exposed to 1  $\mu$ M extracellular clevidine approximates the plasma level in patients receiving a 30 mg dose. Our results indicated that impairments in mitochondrial function and insulin secretion are elicited only by high concentrations of clevidine (> 100  $\mu$ M). This means that clevidine would minimally affect mitochondrial function within the therapeutic concentration range. It is noteworthy, however, that mutations or polymorphisms of DNA polymerase- $\gamma$  were identified in NRTI-treated patients with mitochondrial complications [32]. This suggests that genetic alterations in DNA polymerase- $\gamma$  are not normally deleterious, but that certain conditions such as NRTI treatment may push mitochondrial activity below the clinical threshold, causing pathogenic dysfunction [33]. Differences in genetic susceptibility to mitochondrial toxicity could be one explanation for why a limited proportion of patients receiving clevidine have complications including myopathy.

Clevidine-induced depletion of mtDNA is not restricted to insulin-secreting cells but is also observed in cultured hepatoma cells or muscle tissue from patients [7,26]. Mitochondrial dysfunction in insulin target tissues such as liver and muscle could result in insulin resistance and diabetes [34]. In addition to defects in insulin secretion, decreased sensitivity in insulin target cells can also participate in diabetogenesis in patients receiving clevidine who might have a high susceptibility to mitochondrial toxicity. Interestingly, several reports have shown that NRTI induces intramitochondrial pyrimidine deficiency which may aggravate mtDNA depletion and mitochondrial dysfunction [35,36]. They also discovered that uridine supplementation attenuates steatohepatitis or mitochondrial myopathy induced by NRTI. Further studies concerning the effects of NRTIs on mitochondrial function in different cell types may help us understanding these intractable complications and develop novel antiviral agents.

## Conclusions

In summary, clevidine, used as an antiviral agent against chronic hepatitis B, significantly decreased the

mtDNA copy number at higher doses compared to therapeutic concentration. Mitochondrial dysfunction due to depleted mtDNA and defective ATP synthesis in insulin-releasing cells, consequently led to the impairment of glucose-stimulated insulin secretion. Clevidine-induced mitochondrial dysfunction may contribute to diabetogenesis among clevidine-treated patients who might be more susceptible to mitochondrial toxicity.

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## Author details

<sup>1</sup>Department of Internal Medicine, Yonsei University, Wonju College of Medicine, Wonju, 220-701, Korea. <sup>2</sup>Department of Physiology, Yonsei University, Wonju College of Medicine, Wonju, 220-701, Korea. <sup>3</sup>Department of Basic Science, Yonsei University, Wonju College of Medicine, Wonju, 220-701, Korea. <sup>4</sup>Institute of Lifestyle Medicine, Yonsei University, Wonju College of Medicine, Wonju, 220-701, Korea.

## Authors' contributions

KSP and MYK designed the project. YOJ, XQ, RD, and SX performed the experiments. YOJ, KSP, and MYK wrote the manuscript. CMA prepared a purified clevidine. CHC, CMA, SKB, and IDK contributed to the discussion of the data and the revision of the manuscript. All readers read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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