



The therapeutic efficacy of water-soluble coenzyme Q₁₀ in an experimental model of tacrolimus-induced diabetes mellitus

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Received: August 13, 2019

Revised: September 22, 2019

Accepted: October 1, 2019

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Background/Aims: Coenzyme Q₁₀ (CoQ₁₀) has antioxidant effects and is commercially available and marketed extensively. However, due to its low bioavailability, its effects are still controversial. We developed a water-soluble CoQ₁₀-based micelle formulation (CoQ₁₀-W) and tested it in an experimental model of tacrolimus (TAC)-induced diabetes mellitus (DM).

Methods: We developed CoQ₁₀-W from a glycyrrhizic-carnitine mixed layer CoQ₁₀ micelle preparation based on acyltransferases. TAC-induced DM rats were treated with either lipid-soluble CoQ₁₀ (CoQ₁₀-L) or CoQ₁₀-W for 4 weeks. Their plasma and pancreatic CoQ₁₀ concentrations were measured using liquid chromatography-tandem mass spectrometry. The therapeutic efficacies of CoQ₁₀-W and CoQ₁₀-L on TAC-induced DM were compared using functional and morphological parameters and their effects on cell viability and reactive oxygen species (ROS) production were also evaluated in cultured rat insulinoma cells.

Results: The plasma CoQ₁₀ level was significantly increased in the CoQ₁₀-W group compared to that in the CoQ₁₀-L group. Intraperitoneal glucose tolerance tests and glucose-stimulated insulin secretion revealed that CoQ₁₀-W controlled hyperglycemia and restored insulin secretion significantly better than CoQ₁₀-L. The TAC-mediated decrease in pancreatic islet size was significantly attenuated by CoQ₁₀-W but not by CoQ₁₀-L. TAC-induced oxidative stress and apoptosis were significantly more reduced by CoQ₁₀-W than CoQ₁₀-L. Electron microscopy revealed that CoQ₁₀-W restored TAC-induced attenuation in the number of insulin granules and the average mitochondrial area, unlike CoQ₁₀-L. *In vitro* studies showed that CoQ₁₀-L and CoQ₁₀-W both improved cell viability and reduced ROS production in TAC-treated islet cells to a similar extent.

Conclusions: CoQ₁₀-W has better therapeutic efficacy than CoQ₁₀-L in TAC-induced DM.

Keywords: Coenzyme Q₁₀; Tacrolimus; Diabetes mellitus; Oxidative stress; Apoptosis

INTRODUCTION

Diabetes mellitus (DM) is the most common cause of

end stage renal disease worldwide, and aggravation or development of DM after transplantation is a serious complication in solid organ transplant recipients [1].

Tacrolimus (TAC) is the most popular immunosuppressant but it causes several metabolic complications. These include TAC-induced DM, which is an important adverse effect in organ transplant recipients [2,3]. The mechanisms of TAC-induced DM are not well studied, but direct injury to pancreatic islet cells or impaired insulin signaling by TAC are regarded as the main pathogenesis methods, and oxidative stress plays a significant role in the process of TAC-induced pancreatic islet injury [4,5]. Thus, reducing TAC-induced oxidative stress is one of the important approaches for decreasing TAC-induced DM.

Coenzyme Q₁₀ (CoQ₁₀) is an electron transporter in the electron transport chain (ETC) that transports electrons from ETC complex I and II to complex III [6,7]. With its potential antioxidant effects [8-13], CoQ₁₀ has been commercialized and is widely circulated in health care markets, but its therapeutic effect on diseases such as DM is still controversial due to its low bioavailability. The low bioavailability of CoQ₁₀ is related to its hydrophobic chemical structure, and most of the circulating CoQ₁₀ are in lipid-soluble form.

Thus, improving the bioavailability of CoQ₁₀ is important for optimizing the use of this potential antioxidant. In this study, we developed water-soluble CoQ₁₀ (CoQ₁₀-W) to improve bioavailability and compared its effect with that of lipid-soluble CoQ₁₀ (CoQ₁₀-L) in an experimental model of TAC-induced DM.

METHODS

Development of CoQ₁₀-W

Coenzyme Q₁₀ was purchased from Kaneka Nutrients (Pasadena, TX, USA). Trisodium glycyrrhizinate hydrate was purchased from Tokyo Chemical Industry Co. LTD. (Tokyo, Japan). Eicosapentaenoic acid (EPA) was purchased from Phycoil Biotech Korea, Inc. (Seoul, Korea). EPA was used to improve micelle stability because glycyrrhizin alone is not enough to maintain the micelle. In addition, EPA itself reduces the inflammatory response by inhibiting prostaglandin synthesis. To produce a uniform nano-emulsion (Qmicelle) (Fig. 1A), the following pretreatment is essential. Coenzyme Q₁₀, EPA, and trisodium glycyrrhizinate hydrate (at a concentration of 1 mg/mL each) were dispersed in hot water.

Table 1. Quantification analysis of the nano-emulsion by high performance liquid chromatography

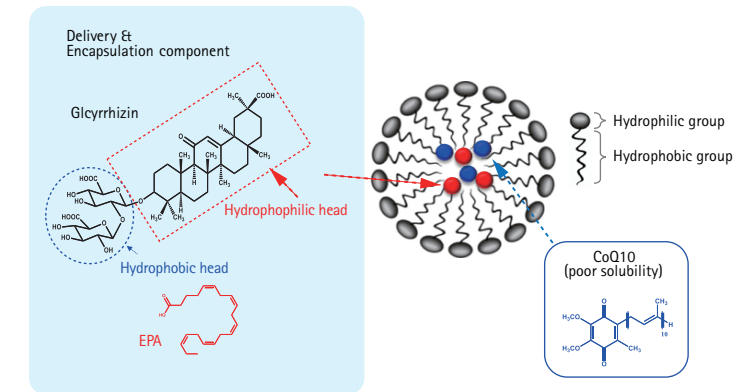
Component	Initial concentration, mg/mL	Final concentration, mg/mL	Proportion of component, %
EPA	1.0	0.24	24
Coenzyme Q ₁₀	1.0	1.0	100
Glycyrrhizinate	1.0	0.6	60

EPA, eicosapentaenoic acid.

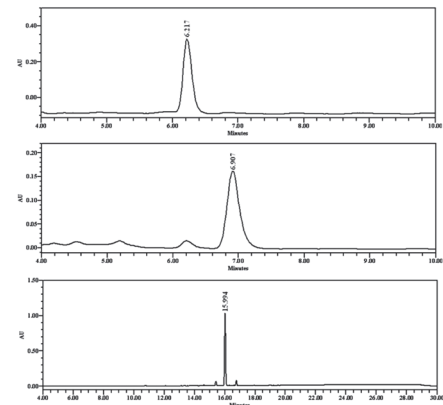
The mixture was pre-homogenized at 10,000 rpm with a homogenizer (Multi-Purpose Homogenizer, YSTRAL, Ballrechten-Dottingen, Germany) until a homogeneous consistency was obtained. An APV2000 microfluidizer processor (SPX Flow, Leeds, UK) was used to produce nano-emulsions (Qmicelle). It has a reservoir capacity of 1,500 mL and can be operated at pressures of up to 2,000 bars. The prepared mixture at 60°C was poured into the fluidizer and Qmicelle was obtained after 10 cycles at a pressure of 1,200 bars. Each component under EPA, coenzyme Q₁₀ and trisodium glycyrrhizinate hydrate, showed single peaks at retention times of 6.0, 6.6, and 15.9 minutes, respectively (Fig. 1B), and their concentrations were 0.24, 1.0 and 0.6 mg/mL (Table 1). We detected homogeneity and size distribution by dynamic light scattering (DLS), and conformed that sizes of the Qmicelles were uniform and distributed in a narrow range around 100 nm (Fig. 1C).

Animals and drugs

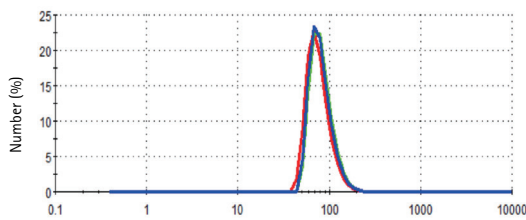
All experimental procedures were reviewed and approved by the animal care and use committee of The Catholic University of Korea (CUMC-2018-0038-03). All the protocols in the study were conducted in strict accordance with their ethical guidelines for animal research. Sprague-Dawley rats (Charles River Technology, Seoul, Korea) with a starting weight of 220 to 230 g were kept in cages (Nalge Co., Rochester, NY, USA) with controlled temperature, humidity, and light in the animal care facility at the Catholic University of Korea. The rats were fed a low-salt diet (0.05% sodium chloride, Research Diets, New Brunswick, NJ, USA) daily. TAC (Prograf, Astellas Pharma, Ibaraki, Japan) was dissolved in olive oil (Sigma-Aldrich, St. Louis, MO, USA), and blended to a final concentration of 1.5 mg/mL. CoQ₁₀ (Chong



A



B



C

— Record 1: KGH005 1 — Record 2: KGH005 2 — Record 3: KGH005 3

Figure 1. The microstructure of coenzyme Q₁₀ (CoQ₁₀)-W. (A) Glycyrrhizin-carnitine mixed layer CoQ₁₀ micelle preparation based on acyltransferases. (B) High performance liquid chromatography profile for the components (1) eicosapentaenoic acid (EPA), (2) coenzyme Q₁₀, and (3) trisodium glycyrrhizinate. Note the single peaks for each component. (C) Size distribution of the nano-emulsion by dynamic light scattering. Note the uniform size around 100 nm. TAC, tacrolimus; TAC + CoQ₁₀-L, TAC cotreatment with lipid-soluble coenzyme Q₁₀; TAC + CoQ₁₀-W, TAC cotreatment with water-soluble coenzyme Q₁₀.

Kun Dang Pharm, Seoul, Korea) was diluted in olive oil (CoQ₁₀-L) or drinking water (CoQ₁₀-W) to a final concentration of 20 mg/kg.

Experimental design

The rats were randomly divided into four groups (n = 9/group). The rats in the vehicle (VH) group received 0.3 mL olive oil once a day subcutaneously. The rats in the TAC, TAC + CoQ₁₀-L and TAC + CoQ₁₀-W groups were treated with TAC (1.5 mg/kg) subcutaneously daily for 4 weeks. The TAC + CoQ₁₀-L and TAC + CoQ₁₀-W groups were co-treated with CoQ₁₀-L or CoQ₁₀-W (20 mg/kg), respectively, orally once a day. We decided the route of administration and concentrations of TAC and CoQ₁₀ based on previous studies [14,15].

Pancreas preservation

Surgical instruments are sterilized before use. Following abdominal incision, phosphate-buffered saline (PBS) was used for *in vivo* perfusion via the abdominal aorta. After ensuring that blood was completely removed from the organs, part of the pancreas was removed for further

analysis. Then the perfusion solution was changed to periodate-lysine-paraformaldehyde, and perfusion was continued for an additional 5 minutes. The remaining portion of the pancreas was collected and wax-embedded for histological studies.

Basic protocol

Following administration of the drug, the diet and weight of the rats were closely monitored for 4 weeks. After 4 weeks of treatment, the rats were placed in a metabolic cage for 24 hours to measure water intake and urine volume. For further analysis, blood and tissue samples were collected from anaesthetized rats a day later. The TAC level in whole blood was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS, Abbott Diagnostics, Abbott Park, IL, USA).

Measurement of CoQ₁₀ in plasma and pancreatic tissue

The concentration of CoQ₁₀ in the plasma and pancreatic tissues was measured by LC-MS/MS analysis. CoQ₁₀ was extracted from the plasma and tissue and quantified

using LC-MS/MS method reported previously [16], with some modifications. Given that CoQ₁₀ is an endogenous substance, all calibration and quality control samples were prepared in a physiological solution (4% bovine serum albumin in PBS). Briefly, 100 μ L aliquots of rat plasma or tissue homogenates treated with 10 μ L of CoQ₁₀ (800 ng/mL, as the internal standard) were extracted by liquid-liquid extraction using 1 mL isopropyl alcohol, followed by LC-MS/MS analysis. The samples were analyzed using a Shimadzu Nexera X2 UPLC (Shimadzu Corporation, Kyoto, Japan) coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation) with an electrospray ionization interface in the positive ion mode. Chromatographic separation was achieved using a Kinetex C₁₈ column (2.1 \times 100 mm, 2.6 μ m; Phenomenex, Seoul, Korea) with a mobile phase consisting of 0.1% formic acid in isopropyl alcohol and methanol at a flow rate of 0.2 mL/min. The total run time was 4 minutes per sample. Quantitation was performed using selected reaction monitoring of the transitions at m/z 863.35 $>$ 197.15 (for CoQ₁₀) and m/z 794.35 $>$ 197.15 (for the internal standard). The calibration curves were linear ($r \geq 0.995$) from 20 to 10,000 ng/mL. The within- and between-batch precision and accuracy were within the acceptable limits of $\pm 15\%$.

Intraperitoneal glucose tolerance test

Rats were fasted for 1 day following treatment with the drugs for 4 weeks before the intraperitoneal glucose tolerance test (IPGTT) was conducted. The rats were injected with 50% dextrose (1.5 g/kg), and blood glucose concentration was measured at 30, 60, 90, and 120 minutes using a glucose analyzer (Accu-Check, Roche Diagnostics, Basel, Switzerland). Thereafter, based on the IPGTT values, trapezoidal estimation was obtained, and the area under the curve of glucose (AUCg) was calculated.

Glucose-stimulated insulin secretion assay

After treating male Sprague-Dawley rats (250 to 300 g) with the drugs according to the *in vivo* experimental design, pancreatic islets were isolated using collagenase digestion as described previously [17]. The islets were incubated in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS and 100 U/mL penicillin at 37°C for 24 hours. The next day, groups of 30

islets were washed with Krebs-Ringer Modified Buffer (KRB; 130 mmol/L sodium chloride [NaCl], 3.6 mmol/L potassium chloride [KCl], 1.5 mmol/L calcium chloride [CaCl₂], 0.5 mmol/L magnesium sulfate [MgSO₄], 0.5 mmol/L potassium dihydrogen phosphate [KH₂PO₄], 2.0 mmol/L sodium bicarbonate [NaHCO₃], and 10 mmol/L 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid [HEPES]) and 2.8 mM glucose (basal) was added. After washing with KRB, the islets were incubated in KRB containing 16.7 mM glucose for 1 hour. The supernatant was collected and the insulin level was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) (Millipore Corp., St. Charles, MO, USA).

Immunohistochemistry

All the immunohistochemistry (IHC) procedures were performed as described previously [18]. The primary antibodies used for IHC were as follows: anti-insulin (I2018, Sigma-Aldrich; 18-0067, Invitrogen, Carlsbad, CA, USA), anti-8-hydroxy-2'-deoxyguanosine (8-OHdG; JaICA, Shizuoka, Japan), and anti-4-hydroxy-2-hexenal (4-HHE, JaICA, Shizuoka, Japan). 4-m samples were incubated with the antibodies for 12 hours at 4°C. A minimum of 20 fields per section was assessed using a color image analyzer (TDI Scope Eye version 3.6 for Windows, JN OptIC Co. Ltd., Seoul, Korea). Briefly, images captured during insulin immunohistochemistry were quantified using the polygon program by measuring the pancreas area that was positively stained for insulin, except for vacuoles, when viewed under $\times 400$ magnification. The measurement of 4-HHE expression was similar to that of insulin, and expressed as percentage of 4-HHE positive area in the islet. The 8-OHdG expression was quantified by counting the number of positive cells per field. Histopathologic analysis was performed on randomly selected fields of the pancreas sectioned by a pathologist blinded to the identity of the treatment groups.

Serum 8-OHdG detection

All procedures were carried out strictly according to the ELISA kit protocol (Cell Biolabs, San Diego, CA, USA).

In situ TUNEL assay

Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using the *in situ* Apoptosis Detection Kit (Millipore, Bill-

erica, MA, USA) as per manufacturer's instructions. In each tissue section, positive cells were analyzed in 20 randomly selected areas. The positive cells or areas were then quantified at $\times 400$ magnification, and the data were analyzed with a color image analyzer (TDI Scope Eye version 3.6 for Windows).

Transmission electron microscopy

Pancreatic tissues were fixed in 2.5% glutaraldehyde (diluted in 0.1 M phosphate buffer), post-fixed with 1% Osmium (VIII) oxide (OsO₄) and then embedded in Epon 812. Ultrathin sections were prepared using the embedded tissues, and the sections were stained with uranyl acetate/lead citrate. A JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan) was used for photography, and 20 randomly selected sites were scanned at $\times 5,000$ magnification. We counted insulin granule number and measured mitochondrial area in the scanned areas using an image analyzer (TDI Scope Eye version 3.6).

In vitro cell culture

The INS-1 rat insulinoma cell line was grown in conditioned RPMI-1640 medium supplemented with 10 nM HEPES, 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol (all from Sigma-Aldrich), 100 IU/mL penicillin, and 100 mg/mL streptomycin (both from Wisent Bio, Saint-Bruno, QC, Canada) in a humidified atmosphere containing 5% CO₂. Cells were plated in culture plates.

Cell viability

A cell counting kit-8 (CCK-8, CK04; Dojindo Molecular Technologies, Rockville, MD, USA) assay kit was used for analyzing cell viability. After plating the INS-1 cells for 1 day, the cells were treated with TAC (40 mg/mL), and same dose of either CoQ₁₀-L (0.1 pg/mL, 1 pg/mL, 10 pg/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL, and 1 μ g/mL) diluted with dimethyl sulfoxide (DMSO) or CoQ₁₀-W (0.1 pg/mL, 1 pg/mL, 10 pg/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL, and 1 μ g/mL) diluted with water for 12 hours. According to the manufacturer's protocol, CCK-8 or propidium iodide (PI; 556463, BD Biosciences, San Jose, CA, USA) solution was added for 2 hours. Following CCK-8 treatment, the absorbance was measured at 450 nm using a Versa Max ELISA Reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry

To assess reactive oxygen species (ROS) production, we performed flow cytometry. INS-1 cells were plated and pre-incubated in an incubator at 37°C for 24 hours. After 24 hours, the culture medium was changed to serum-free medium containing TAC (40 mg/mL), TAC (40 mg/mL) + CoQ₁₀-L (10 ng/mL) or TAC + CoQ₁₀-W (10 ng/mL). The method followed has been described previously [19].

Statistical analysis

The data are expressed as mean \pm standard error (SE) of at least three independent experiments. Multiple comparisons between different groups were carried out by one-way analysis of variance with Bonferroni's *post hoc* test using IBM SPSS Statistics version 24 (IBM, Armonk, NY, USA). Results with $p < 0.05$ were considered statistically significant.

RESULTS

Comparison of the basic parameters of CoQ₁₀-L and CoQ₁₀-W in TAC-induced DM rats

TAC treatment for 4 weeks significantly reduced the body weight compared to VH treatment (48 ± 6 g vs. 67 ± 3 g, $p < 0.05$). These changes were restored in the TAC + CoQ₁₀-L and TAC + CoQ₁₀-W groups (48 ± 6 g vs. 62 ± 1 g and 65 ± 2 g, $p < 0.05$). Water intake was increased in the TAC group compared with the VH group (49 ± 6 mL vs. 30 ± 2 mL, $p < 0.05$). In the TAC + CoQ₁₀-L and TAC + CoQ₁₀-W groups, water intake was lower than in the TAC group (35 ± 1 mL and 39 ± 4 mL vs. 49 ± 6 mL, $p < 0.05$). The concentration of TAC in whole blood was not affected by CoQ₁₀-L or CoQ₁₀-W treatment (9.4 ± 4 ng/mL vs. 9.3 ± 2 or 9.3 ± 3 ng/mL, $p > 0.05$).

Comparison of the plasma and pancreas CoQ₁₀ levels with CoQ₁₀-L and CoQ₁₀-W treatments of TAC-induced DM rats

Plasma CoQ₁₀ levels in the VH and TAC groups were 39 ± 7 and 25 ± 8 ng/mL, respectively. The plasma CoQ₁₀ level (Fig. 2A) was significantly higher in the TAC + CoQ₁₀-W group than the TAC + CoQ₁₀-L group (956 ± 97 ng/mL vs. 577 ± 88 ng/mL, $p < 0.05$). The pancreatic CoQ₁₀ level (Fig. 2B) was lower in the TAC group than in the VH group (59 ± 8 ng/mL vs. 103 ± 26 ng/mL, p

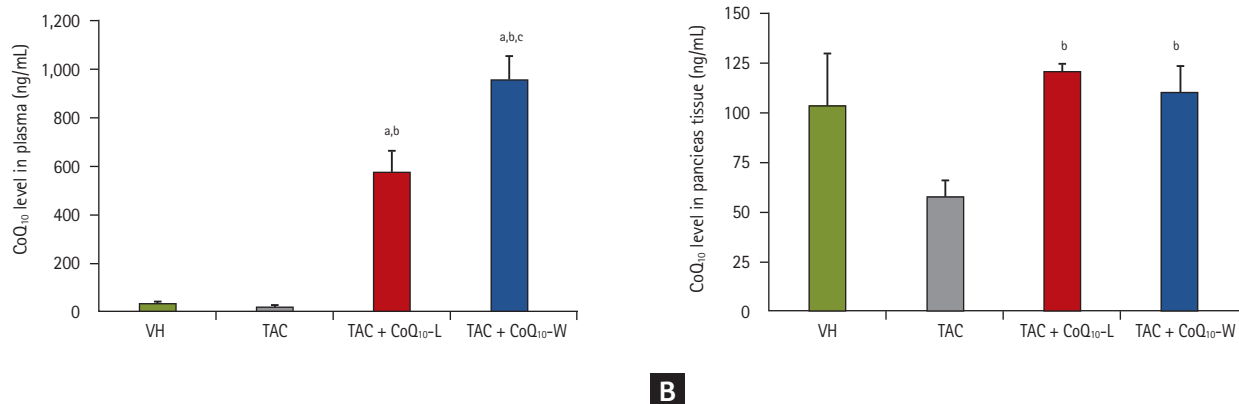


Figure 2. Comparison of plasma and pancreas coenzyme Q₁₀ (CoQ₁₀) levels in CoQ₁₀-L and CoQ₁₀-W in tacrolimus (TAC)-induced diabetes mellitus rats. (A) Concentrations of CoQ₁₀ in plasma and (B) pancreas tissue were measured by liquid chromatography-tandem mass spectrometry analysis. Data are presented as mean ± standard error (n = 9). One-way analysis of variance was used to analyze the data. VH, vehicle; TAC + CoQ₁₀-L, TAC cotreatment with lipid-soluble coenzyme Q₁₀; TAC + CoQ₁₀-W, TAC cotreatment with water-soluble coenzyme Q₁₀. ^a*p* < 0.05 vs. VH. ^b*p* < 0.05 vs. TAC. ^c*p* < 0.05 vs. TAC + CoQ₁₀-L.

< 0.05). Treatment with either TAC + CoQ₁₀-L or TAC + CoQ₁₀-W increased pancreatic CoQ₁₀ levels compared with the TAC only group (121 ± 4 ng/mL and 110 ± 14 ng/mL vs. 59 ± 8 ng/mL, *p* < 0.05). However, there was no significant difference between the two groups.

Comparison of blood glucose levels with CoQ₁₀-L and CoQ₁₀-W treatments of TAC-induced DM rats

After the 4-week drug-treatment regimen, we performed a IPGTT to evaluate the changes in blood glucose level. Thirty minutes after injecting 50% dextrose, blood glucose was significantly higher in the TAC group than the VH group (434 ± 9 ng/mL vs. 322 ± 16 mg/dL, *p* < 0.05). Unlike TAC + CoQ₁₀-L treatment (430 ± 6 mg/dL), TAC + CoQ₁₀-W treatment (379 ± 14 mg/dL) decreased the blood glucose level relative to TAC for 120 minutes (Fig. 3A). The increase in AUC_g values detected by IPGTT demonstrated that DM was successfully induced by TAC (273 ± 19 mg/dL/min vs. 162 ± 18 mg/dL/min, *p* < 0.05). The TAC + CoQ₁₀-L group had no significant difference in AUC_g compared with the TAC group (241 ± 23 vs. 273 ± 19 mg/dL/min, *p* > 0.05). The decrease was markedly higher in the TAC + CoQ₁₀-W group (170 ± 12 mg/dL/min, *p* < 0.05) (Fig. 3B).

Comparison of insulin secretion with CoQ₁₀-L and CoQ₁₀-W treatments of TAC-induced DM rats

Insulin secretion was significantly reduced by TAC treat-

ment compared to VH (11 ± 0.6 ng/mL vs. 25 ± 1 ng/mL, *p* < 0.05) (Fig. 4). CoQ₁₀-W increased insulin secretion (15 ± 0.5 ng/mL, *p* < 0.05) unlike CoQ₁₀-L (11 ± 0.2 ng/mL, *p* > 0.05).

Comparison of islet size with CoQ₁₀-L and CoQ₁₀-W treatments of TAC-induced DM rats

Immunohistochemical staining for insulin showed that the islet sizes in the TAC group and TAC + CoQ₁₀-L group were lower than that in the VH group (8 ± 2 and 9 ± 1 vs. 16 ± 2 μm² × 10³, *p* < 0.05). However, the islet size in the TAC + CoQ₁₀-W group (14 ± 1 μm² × 10³) was increased compared with the TAC and the TAC + CoQ₁₀-L groups (*p* < 0.05) (Fig. 5).

Comparison of oxidative stress with CoQ₁₀-L and CoQ₁₀-W treatments of TAC-induced DM rats

Immunohistochemical staining for the oxidative stress markers 8-OHdG (Fig. 6A and 6B), and 4-HHE (Fig. 6C and 6D), and ELISA for serum 8-OHdG (Fig. 6E) were used to identify the anti-oxidative effects. The number of 8-OHdG-positive cells was significantly increased in the TAC group compared with the VH group (140 ± 10 per field vs. 3 ± 1 per field, *p* < 0.05). CoQ₁₀-W (83 ± 6 per field) significantly decreased the expression of 8-OHdG unlike CoQ₁₀-L (138 ± 6 per field). The expression of 4-HHE was significantly higher in the TAC group than in the VH group (15% ± 1% vs. 0.3% ± 1%, *p* < 0.05). CoQ₁₀-W (6% ± 1%) significantly decreased the expression of 4-HHE (*p* <

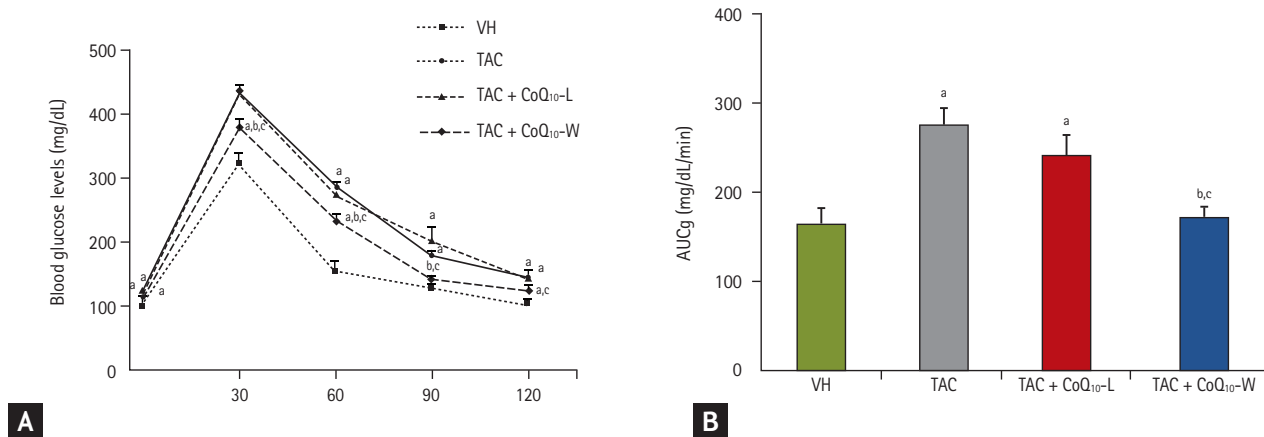


Figure 3. Comparison of blood glucose with coenzyme Q₁₀ (CoQ₁₀)-L and CoQ₁₀-W treatments of tacrolimus (TAC)-induced diabetes mellitus rats. (A) Blood glucose levels were measured using intraperitoneal glucose tolerance test (IPGTT), and the (B) AUCg was calculated from IPGTT values. Data are presented as mean ± standard error (n = 9). Scale bar = 100 μm. One-way analysis of variance was used to analyze the data. VH, vehicle; TAC + CoQ₁₀-L, TAC cotreatment with lipid-soluble coenzyme Q₁₀; TAC + CoQ₁₀-W, TAC cotreatment with water-soluble coenzyme Q₁₀; AUCg, area under the curve of glucose. ^a*p* < 0.05 vs. VH. ^b*p* < 0.05 vs. TAC. ^c*p* < 0.05 vs. TAC + CoQ₁₀-L.

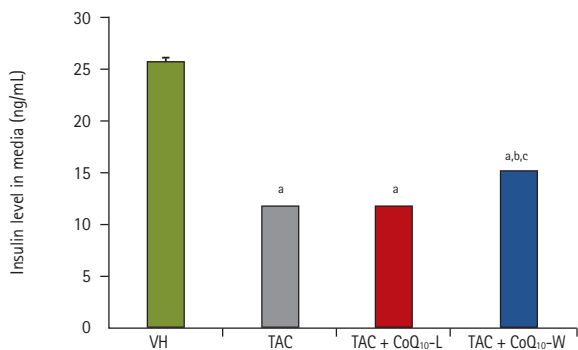


Figure 4. Comparison of insulin secretion after glucose-stimulated insulin secretion (GSIS) with coenzyme Q₁₀ (CoQ₁₀)-L and CoQ₁₀-W treatments of tacrolimus (TAC)-induced diabetes mellitus rats. enzyme-linked immunosorbent assay was used to measure insulin secretion after GSIS. Data are presented as mean ± standard error (n = 9). Scale bar = 100 μm. One-way analysis of variance was used to analyze the data. VH, vehicle; TAC + CoQ₁₀-L, TAC cotreatment with lipid-soluble coenzyme Q₁₀; TAC + CoQ₁₀-W, TAC cotreatment with water-soluble coenzyme Q₁₀. ^a*p* < 0.05 vs. VH. ^b*p* < 0.05 vs. TAC. ^c*p* < 0.05 vs. TAC + CoQ₁₀-L.

0.05) while CoQ₁₀-L (14% ± 1%) did not. Similar changes were observed in serum 8-OHdG level (Fig. 6E).

Comparison of apoptosis in pancreatic islets with CoQ₁₀-L and CoQ₁₀-W treatments of TAC-induced DM rats

Four weeks of TAC treatment also induced apoptosis in the pancreas. As shown in Fig. 7, the number of TUNEL-positive cells was significantly higher in the TAC group than in the VH group (2.4 ± 0.3 cells per islet vs. 0 ± 0 cells per islet, *p* < 0.05). CoQ₁₀-W decreased TUNEL-positive cells compared with TAC (0.2 ± 0.2 cells per islet vs. 2.4 ± 0.3 cells per islet, *p* < 0.05), while CoQ₁₀-L (1.8 ± 0.2 cells per islet) did not (Fig. 7).

Comparison of the mitochondrial area and insulin granule with CoQ₁₀-L and CoQ₁₀-W treatments of TAC-induced DM rats

Electron microscopy was used to evaluate the changes in the mitochondrial area and insulin granule number. The average mitochondrial area was significantly lower in the TAC and TAC + CoQ₁₀-L groups than in the VH group (0.05 ± 0.006 and 0.08 ± 0.08 vs. 0.4 ± 0.03 per field, *p* < 0.05). However, the numbers were significantly (*p* < 0.05) higher in the TAC+CoQ₁₀-W group (0.2 ± 0.02 per field) (Fig. 8A and 8C). Changes in insulin granule numbers were similar to the changes in mitochondrial area (Fig. 8A and 8B).

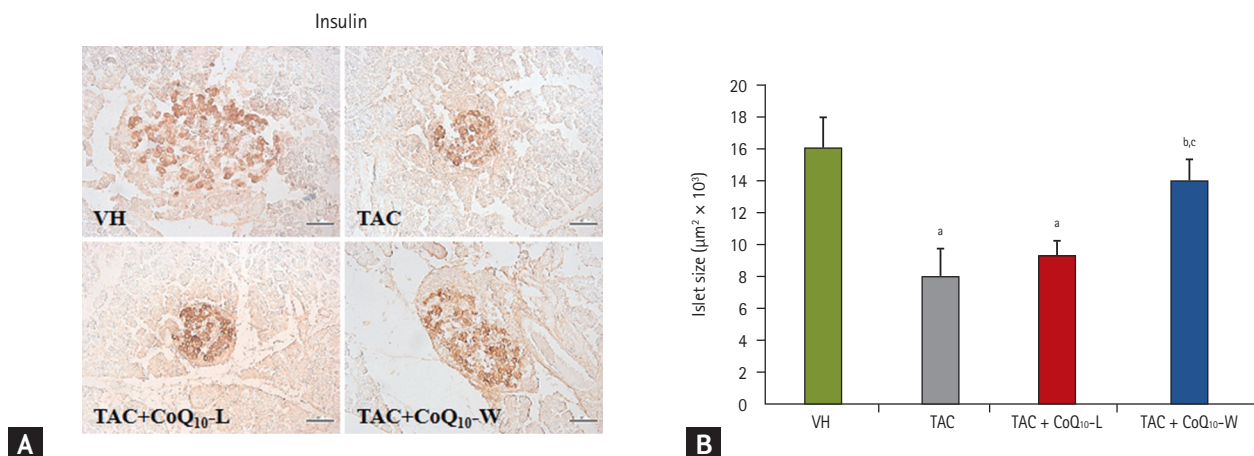


Figure 5. Comparison of islet size with coenzyme Q₁₀ (CoQ₁₀)-L and CoQ₁₀-W treatments of tacrolimus (TAC)-induced diabetes mellitus rats using immunohistochemical analysis in tissues section. (A) Representative images of insulin staining in pancreatic sections. (B) Quantitative analysis of beta cell area. Data are presented as mean ± standard error (n = 9). Scale bar = 100 µm. One-way analysis of variance was used to analyze the data. VH, vehicle; TAC + CoQ₁₀-L, TAC cotreatment with lipid-soluble coenzyme Q₁₀; TAC + CoQ₁₀-W, TAC cotreatment with water-soluble coenzyme Q₁₀. ^a*p* < 0.05 vs. VH. ^b*p* < 0.05 vs. TAC. ^c*p* < 0.05 vs. TAC + CoQ₁₀-L.

Comparison of cell viability with CoQ₁₀-L and CoQ₁₀-W treatments of TAC-induced INS cell injury

We demonstrated the protective effect of CoQ₁₀-L and CoQ₁₀-W against cell injury during TAC treatment in INS-1, a pancreatic beta cell line. At the different doses tested, both TAC + CoQ₁₀-L and TAC + CoQ₁₀-W increased cell viability by approximately 2.8-fold compared to TAC (*p* < 0.05). However, there were no differences between the tested doses of TAC + CoQ₁₀-L and TAC + CoQ₁₀-W (*p* > 0.05) (Fig. 9).

Comparison of ROS production with CoQ₁₀-L and CoQ₁₀-W treatments of TAC-induced INS cell injury

To compare the protective effect of CoQ₁₀-L and CoQ₁₀-W on oxidative stress, we performed flow cytometry using INS-1 cells. TAC significantly increased ROS production compared to the VH (*p* < 0.05). Both CoQ₁₀-L and CoQ₁₀-W reduced TAC-induced ROS production; though there was no significant difference between the two groups (*p* > 0.05) (Fig. 10).

DISCUSSION

This study investigated whether CoQ₁₀-W provides better protection against TAC-induced pancreatic beta cell

dysfunction than conventional CoQ₁₀ (CoQ₁₀-L). The results of our study clearly demonstrate that CoQ₁₀-W increases insulin secretion, and thus lowers blood glucose in TAC-induced diabetes. In addition, CoQ₁₀-W reduced TAC-induced oxidative stress and apoptosis in the pancreas more than CoQ₁₀-L. This finding suggests that improving the bioavailability using CoQ₁₀-W may overcome the limitations of CoQ₁₀-L and render it more effective as an anti-diabetic and anti-oxidative agent.

Until now, several approaches have been used for improving the bioavailability of CoQ₁₀ [20-23] that can be summarized in two ways as follows: One is to increase the dissolution rate of the drug, and the other is to promote absorption of the drug by optimizing the digestion process. We used the latter method of optimizing the digestion process, to improve drug absorption. The CoQ₁₀-W used in this study consists of a glycyrrhizin-carnitine mixed layer CoQ₁₀ micelle preparation based on acyltransferases as shown in Fig. 1. This formula improved the absorption of CoQ₁₀ by using the natural extract-glycyrrhizin as the carrier, and the heat of fusion which converts the CoQ₁₀ from hydrophobic to hydrophilic. In addition, this formulation has the advantage of retaining the original chemical structure of CoQ₁₀ and eliminating the formation of toxic substances during the synthesis and conversion processes. The

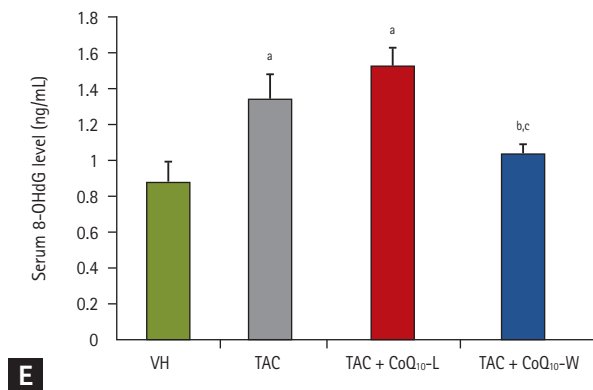
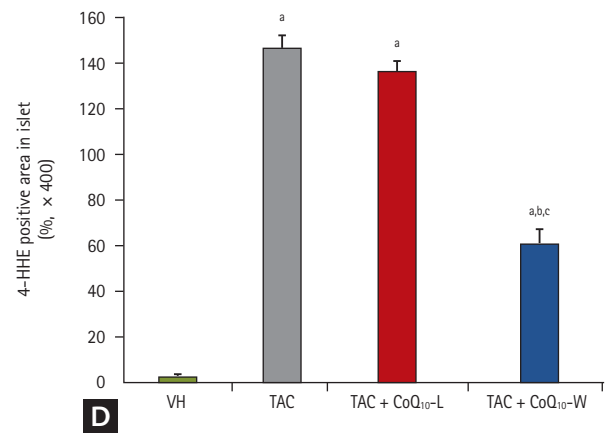
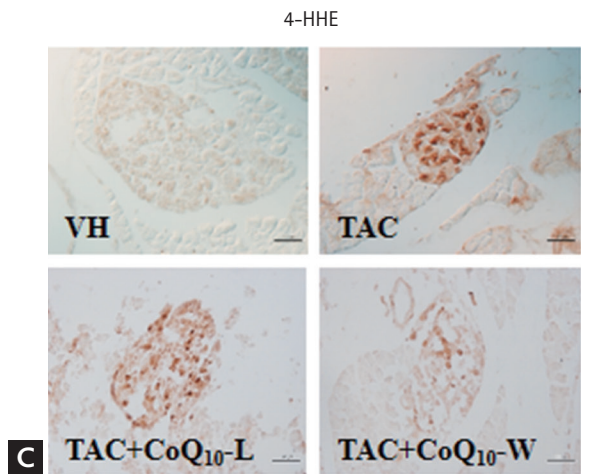
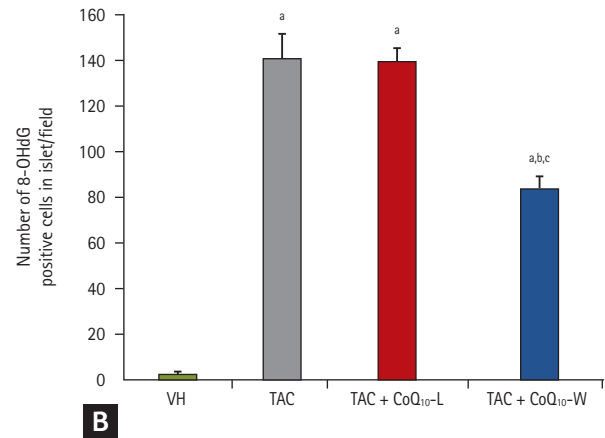
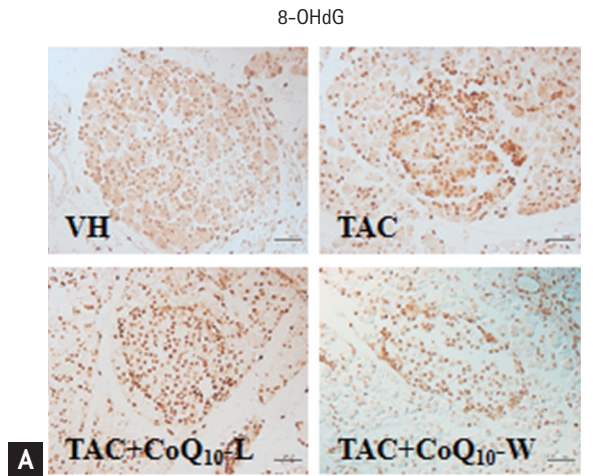


Figure 6. Comparison of oxidative stress with coenzyme Q₁₀ (CoQ₁₀)-L and CoQ₁₀-W treatments of tacrolimus (TAC)-induced diabetes mellitus rats using immunohistochemical analysis in tissues sections. Representative images and quantification of immunohistochemistry for (A, B) 8-hydroxy-2'-deoxyguanosine (8-OHdG) and (C, D) 4-hydroxy-2-hexenal (4-HHE) using rat tissue sections. (E) Representative quantification of 8-OHdG enzyme-linked immunosorbent assay on rat serum. Scale bar = 100 μm (all panels). Data are presented as mean ± standard error (n = 8). One-way analysis of variance was used to analyze the data. VH, vehicle; TAC + CoQ₁₀-L, TAC cotreatment with lipid-soluble coenzyme Q₁₀; TAC + CoQ₁₀-W, TAC cotreatment with water-soluble coenzyme Q₁₀. ^a*p* < 0.05 vs. VH. ^b*p* < 0.05 vs. TAC. ^c*p* < 0.05 vs. TAC + CoQ₁₀-L.

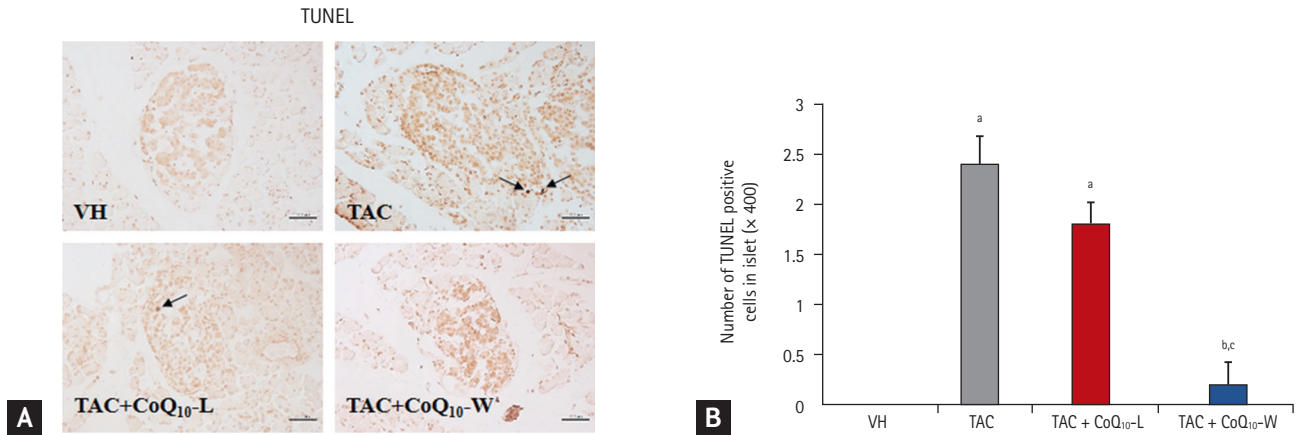


Figure 7. Comparison of apoptosis in pancreatic islets with coenzyme Q₁₀ (CoQ₁₀)-L and CoQ₁₀-W treatments of tacrolimus (TAC)-induced diabetes mellitus in rats. Representative (A) images and (B) quantification of TUNEL staining. Arrows indicate TUNEL positive cells. Scale bar = 100 μm (all panels). Data are presented as mean ± standard error (n = 9). One-way analysis of variance was used to analyze the data. VH, vehicle; TAC + CoQ₁₀-L, TAC cotreatment with lipid-soluble coenzyme Q₁₀; TAC + CoQ₁₀-W, TAC cotreatment with water-soluble coenzyme Q₁₀; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick-end labeling. ^a*p* < 0.05 vs. VH. ^b*p* < 0.05 vs. TAC. ^c*p* < 0.05 vs. TAC + CoQ₁₀-L.

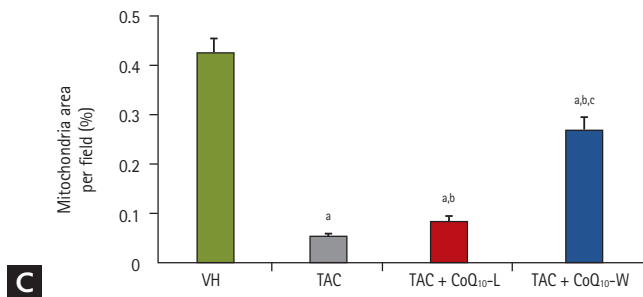
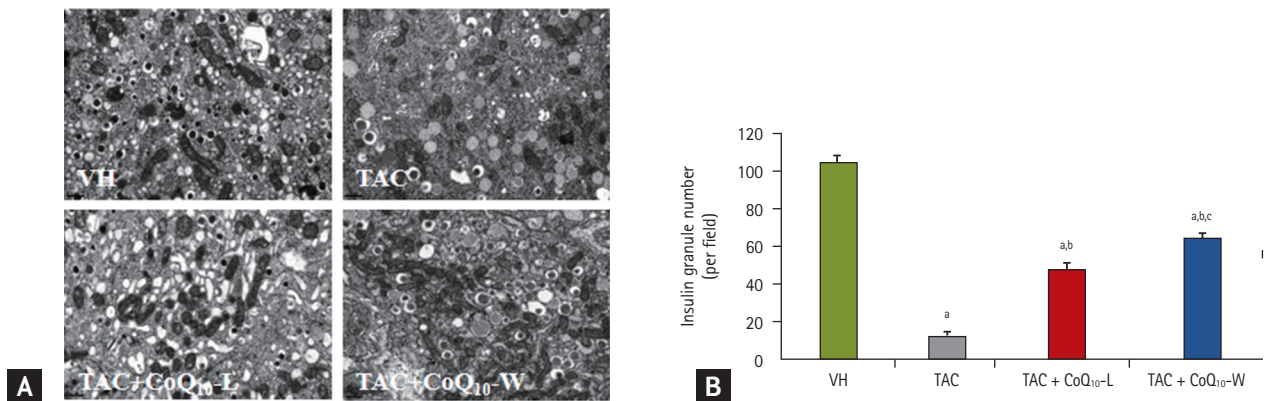


Figure 8. Comparison of the mitochondrial area and insulin granules by electron microscopy (×5,000 magnification) with coenzyme Q₁₀ (CoQ₁₀)-L and CoQ₁₀-W treatments of tacrolimus (TAC)-induced diabetes mellitus rats. (A) Representative images of mitochondria and insulin granules, and (B, C) quantification using electron microscopy. Scale bar = 500 nm. Data are presented as mean ± standard error (n = 9). One-way analysis of variance was used to analyze the data. VH, vehicle; TAC + CoQ₁₀-L, TAC cotreatment with lipid-soluble coenzyme Q₁₀; TAC + CoQ₁₀-W, TAC cotreatment with water-soluble coenzyme Q₁₀. ^a*p* < 0.05 vs. VH. ^b*p* < 0.05 vs. TAC. ^c*p* < 0.05 vs. TAC + CoQ₁₀-L.

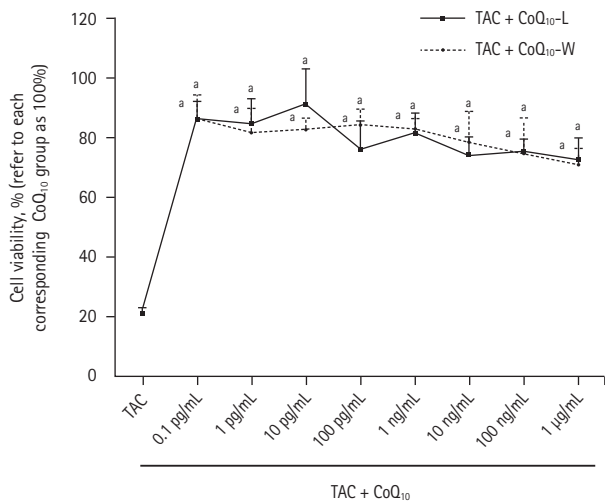


Figure 9. Comparison of cell viability with coenzyme Q₁₀ (CoQ₁₀)-L and CoQ₁₀-W treatments of tacrolimus (TAC)-induced insulinoma (INS) cell injury. Cell viability was evaluated with a cell counting kit 8 (CCK-8) assay. Data are presented as mean ± standard error (n = 5). One-way analysis of variance was used to analyze the data. TAC + CoQ₁₀-L, TAC cotreatment with lipid-soluble coenzyme Q₁₀; TAC + CoQ₁₀-W, TAC cotreatment with water-soluble coenzyme Q₁₀. ^ap < 0.05 vs. TAC.

composition of CoQ₁₀ in the solution may be affected by high pressure or high temperature during the manufacture of CoQ₁₀-W. Thus, we confirmed the homogeneity and uniform size of the micelles by high performance liquid chromatography after preparation as shown Fig. 1. With this formulation, we achieved higher plasma CoQ₁₀ levels (1.7 times) than with CoQ₁₀-L.

We evaluated whether CoQ₁₀-W had improved effects on glucose-lowering and insulin secretion compared to CoQ₁₀-L in an experimental model of TAC-induced DM. In this study, we found that CoQ₁₀-W significantly reduced TAC-induced hyperglycemia, while CoQ₁₀-L did not. In addition, glucose-stimulated insulin secretion (GSIS) revealed that CoQ₁₀-W significantly restored the TAC-induced decrease in insulin secretion, unlike CoQ₁₀-L. These results suggest that CoQ₁₀-W is more effective than CoQ₁₀-L at lowering hyperglycemia, and in preserving insulin secretion in TAC-induced DM, and this is related to the increased bioavailability of CoQ₁₀-W. However, pancreatic CoQ₁₀ levels were not significantly different in the two groups. Thus, the improved control of hyperglycemia or insulin secretion in the CoQ₁₀-W group compared with the CoQ₁₀-L group seems to be related to a systemic antioxidant effect rather

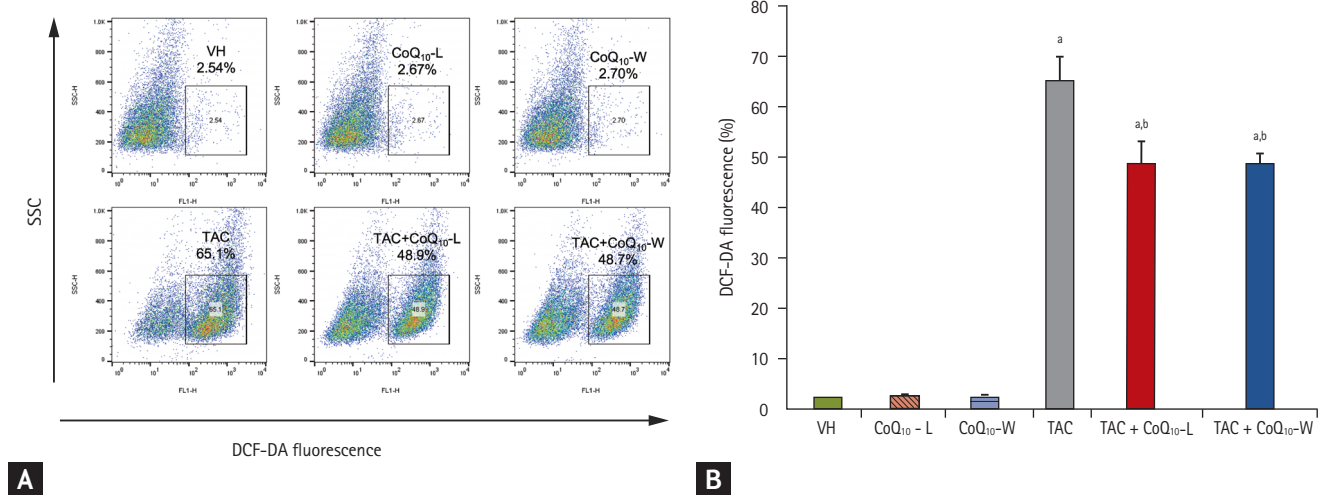


Figure 10. Comparison of reactive oxygen species (ROS) production with coenzyme Q₁₀ (CoQ₁₀)-L and CoQ₁₀-W treatments of tacrolimus (TAC)-induced insulinoma (INS) cell injury. Intracellular ROS production was measured using flow cytometry. (A) 2', 7'-Dichlorofluorescein-diacetate (DCF-DA) was used as a probe to evaluate alterations in intracellular ROS, and (B) quantitative analysis of ROS production was carried out. Data are presented as mean ± standard error (n = 9). One-way analysis of variance was used to analyze the data. VH, vehicle; TAC + CoQ₁₀-L, TAC cotreatment with lipid-soluble coenzyme Q₁₀; TAC + CoQ₁₀-W, TAC cotreatment with water-soluble coenzyme Q₁₀; SSC-H, side scatter height. ^ap < 0.05 vs. VH. ^bp < 0.05 vs. TAC.

er than its local effect on pancreatic tissue. This presumption may be supported by the lack of difference in cell survival or ROS production in *in vitro* studies of the two groups.

It is well known that oxidative stress [4,24,25] and apoptosis [25-27] are common mechanisms of TAC-induced DM. To explain the improved hyperglycemia control by CoQ₁₀-W, we evaluated the antioxidant and anti-apoptotic effects of CoQ₁₀-W and CoQ₁₀-L in TAC-induced pancreatic islet injury. The results of our study clearly demonstrate that CoQ₁₀-W reduced both, the TAC-induced markers of oxidative stress (8-OHdG in tissue and serum, 4-HHE in tissue), and TAC-induced apoptosis to a greater extent than CoQ₁₀-L. These findings confirm that CoQ₁₀-W has better antioxidant and anti-apoptotic effects than CoQ₁₀-L on TAC-induced pancreas injury, and this may explain the better hyperglycemia control of CoQ₁₀-W than CoQ₁₀-L.

Oxidative stress is closely related to the quantity and quality of mitochondria [28,29]. High-quality mitochondria protect cells against oxidative stress and prevent apoptosis [29]. In our data, CoQ₁₀-L increased number of insulin granules compared with the TAC group, with CoQ₁₀-W producing a further increase as shown by electron microscopy. Furthermore, CoQ₁₀-W increased the average mitochondria area over that seen in the TAC group. Although CoQ₁₀-L also restored the loss of mitochondria, it was significantly less effective than CoQ₁₀-W. These findings suggest that the improved antioxidant capacity of CoQ₁₀-W offers a more effective remedy for islet and mitochondrial damage than CoQ₁₀-L in the TAC-induced DM model.

Our study has some limitations. First, the CoQ₁₀-W group had a similar CoQ₁₀ level in the pancreas as the CoQ₁₀-L group, and *in vitro* study revealed that viability and ROS production of islet cells in the CoQ₁₀-L and CoQ₁₀-W groups were not significantly. Further studies are needed to improve the action of CoQ₁₀-W at the cellular level. Second, we compared the CoQ₁₀ levels at the end of our study. However, endogenous substances such as CoQ₁₀ follow the circadian rhythm. Thus, pharmacokinetic and pharmacodynamic studies are needed to determine whether CoQ₁₀-W has better bioavailability than CoQ₁₀-L. Third, we did not include the VH of nano-emulsion without CoQ₁₀. Therefore, there is a concern that that anti-inflammatory effect of EPA in na-

no-emulsion may affect that of CoQ₁₀-W. In this point, we previously tested the anti-inflammatory effect of EPA in rheumatoid arthritis model, and found that EPA in nano-emulsion did not affect the anti-inflammatory effect of CoQ₁₀ (data not shown). Thus, the anti-inflammatory effect of EPA in nano-emulsion may be ruled out in our study.

In summary, the improved CoQ₁₀ bioavailability using CoQ₁₀-W may provide more benefit than conventional CoQ₁₀ in TAC-induced diabetes. We expect that adding CoQ₁₀-W TAC treatments can effectively reduce TAC-induced oxidative stress in clinical practice.

KEY MESSAGE

1. Water-soluble coenzyme Q₁₀ (CoQ₁₀) improves bioavailability compared to conventional lipid-soluble CoQ₁₀.
2. Water-soluble CoQ₁₀ reduces oxidative stress more effectively than lipid-soluble CoQ₁₀.
3. Treatment with a combination of water-soluble CoQ₁₀ and tacrolimus provides effect protection against tacrolimus-induced pancreatic islet injury.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

This study was supported by a grant of the Korean Health Technology R&D Project, Ministry for Health and Welfare, Republic of Korea (HI14C3417) and the Bio & Medical Technology Development Program of the National Research Foundation (NRF) & funded by the Korean government (MSIT) (NRF-2019M3A9A8064802).

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