



# Water-soluble coenzyme Q<sub>10</sub> provides better protection than lipid-soluble coenzyme Q<sub>10</sub> in a rat model of chronic tacrolimus nephropathy

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**Background/Aims:** Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>), is a promising antioxidant; however, low bioavailability owing to lipid-solubility is a limiting factor. We developed water-soluble CoQ<sub>10</sub> (CoQ<sub>10</sub>-W) and compared its effects with conventional lipid-soluble CoQ<sub>10</sub> (CoQ<sub>10</sub>-L) in an experimental model of chronic tacrolimus (Tac) nephropathy.

**Methods:** CoQ<sub>10</sub>-W was developed from a glycyrrhizic-carnitine mixed layer CoQ<sub>10</sub> micelle based on acyltransferases. Chronic nephropathy was induced in rats with 28-day Tac treatment; they were concomitantly treated with CoQ<sub>10</sub>-L or CoQ<sub>10</sub>-W. CoQ<sub>10</sub> level in plasma and kidney were measured using liquid chromatography–mass spectrometry. CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L effects on Tac-induced nephropathy were assessed in terms of renal function, histopathology, oxidative stress, and apoptotic cell death. Their effects on cell viability and reactive oxygen species (ROS) production were assessed in cultured proximal tubular cells, human kidney 2 (HK-2) cells.

**Results:** The plasma CoQ<sub>10</sub> level was significantly higher in the CoQ<sub>10</sub>-W group than in the CoQ<sub>10</sub>-L group. Tac treatment caused renal dysfunction, typical pathologic lesions, and oxidative stress markers. Serum creatinine was restored in the Tac + CoQ<sub>10</sub>-L or CoQ<sub>10</sub>-W groups compared with that in the Tac group. CoQ<sub>10</sub>-W administration reduced oxidative stress and apoptosis markers. Mitochondrial ultrastructure assessment revealed that the addition of CoQ<sub>10</sub>-L or CoQ<sub>10</sub>-W with Tac increased mitochondrial size and number than Tac treatment alone. In vitro investigations revealed that both CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W improved cell viability and reduced ROS production in the Tac-induced HK-2 cell injury.

**Conclusions:** CoQ<sub>10</sub>-W has a better therapeutic effect in Tac-induced renal injury than conventional CoQ<sub>10</sub>-L, possibly associated with improved CoQ<sub>10</sub> bioavailability.

**Keywords:** Tacrolimus; Coenzyme Q<sub>10</sub>; Water-soluble coenzyme Q<sub>10</sub>; Kidney; Oxidative stress

## INTRODUCTION

Tacrolimus (Tac), a calcineurin inhibitor, is the most popular regimen in current clinical practice because this regimen can significantly reduce acute rejection rates of transplants and provide excellent early outcomes. In spite of excellent immunosuppression, Tac has considerable side effects that may hamper long-term kidney graft and patient survival, and cause major additional morbidity [1]. The pathogenesis is multifactorial, and oxidative stress has been proposed as a common mechanism of Tac-induced renal injury [2-4]. Thus, reducing Tac-induced oxidative stress is a promising approach to improving therapeutic outcomes.

Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>), a lipid-soluble molecule derived mainly from endogenous synthesis [5], with the highest levels in the heart, liver, kidney, and pancreas, acts as an antioxidant by functioning as an electron carrier in mitochondrial oxidative phosphorylation [6]. Because lipid-soluble CoQ<sub>10</sub> (CoQ<sub>10</sub>-L) has important functions in the body and people with some diseases have reduced levels of this substance, several studies have focused on investigating whether CoQ<sub>10</sub>-L supplements might have health benefits [7].

Therapeutic applications of CoQ<sub>10</sub> are greatly limited by its poor bio-availability, due to its lack of solubility in aqueous media. A recent study demonstrated that, in rats, only 3% of orally administered CoQ<sub>10</sub> can be absorbed [8]. Supplementation with enhanced bio-availability of CoQ<sub>10</sub> formulation has been thought to be more beneficial, especially for situations in which adequate CoQ<sub>10</sub> production is adversely affected [9]. Several advancements have been made to enhance the bioavailability of CoQ<sub>10</sub> using various approaches like size reduction, solubility enhancement (by solid dispersion, prodrug, complexation, ionization) and use of novel drug carriers such as liposomes, microspheres, nanoparticles, nanoemulsions, and self-emulsifying systems [10].

Thus, we developed a water soluble CoQ<sub>10</sub> (CoQ<sub>10</sub>-W) as better uptake may be the first step in improving its biological availability and shows more benefit effects than CoQ<sub>10</sub>-L [11-15].

## METHODS

### Development of CoQ<sub>10</sub>-W

CoQ<sub>10</sub> 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), the following pretreatment is essential. Coenzyme Q<sub>10</sub>, EPA, and trisodium glycyrrhizinate hydrate (at a concentration of 1 mg/mL each) were dispersed in hot water 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, CoQ<sub>10</sub>, and trisodium glycyrrhizinate hydrate, showed single peaks at retention times of 6.0, 6.6, and 15.9 minutes, respectively, and their concentrations were 0.24, 1.0, and 0.6 mg/mL. 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.

### Animals and drugs

All animal experiments in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals, Catholic University of Korea (CUMC-2018-0340-01), and all procedures in this study were performed in accordance with the ethical guidelines for animal studies. Eight-week-old male Sprague Dawley rats (Charles River Technology, Seoul, Korea) that initially weighed 220 to 230 g were housed in cages (Nalge Co., Rochester, NY, USA) in a temperature- and light-controlled environment at the Catholic University of Korea's animal care facility. The rats received a low-

salt diet (0.05% sodium; Teklad Premier, Madison, WI, USA).

### Experiments and designs

The primary goals of this study were to determine if CoQ<sub>10</sub>-W has better protective effects than CoQ<sub>10</sub>-L in Tac-induced renal injury. After acclimation for 1 week, weight-matched rats were randomized into four groups, nine per group, as follows.

- (1) Vehicle (VH) group: rats received a daily subcutaneous injection of olive oil (0.3 mg/kg) and oral administration of sterile water for 4 weeks.
- (2) Tac group: rats received a daily subcutaneous injection of Tac (1.5 mg/kg).
- (3) Tac + CoQ<sub>10</sub>-L group: rats received a daily subcutaneous injection of Tac (1.5 mg/kg) and oral administration of CoQ<sub>10</sub>-L (20 mg/kg) for 4 weeks.
- (4) Tac + CoQ<sub>10</sub>-W group: rats received a daily subcutaneous injection of Tac (1.5 mg/kg) and oral administration of CoQ<sub>10</sub>-W (20 mg/kg) for 4 weeks.

We decided on the route of administration and the concentration based on previous studies [16,17].

Rats were pair-fed, and their body weight was monitored daily. After the 4-week treatment period, animals were housed individually in metabolic cages, and their water intake and urine volume were measured over 24 hours. On the following day, animals were anesthetized, and blood samples and tissue specimens were obtained for further analysis. Serum creatinine was measured using a quantitative enzyme colorimetric method (Stanbio Laboratory, Boerne, TX, USA).

### Measurement of Tac level and CoQ<sub>10</sub> concentration

Tac level in whole-blood was measured using liquid chromatography-tandem mass spectrometry (Abbott Diagnostics, Abbott Park, IL, USA). Content of CoQ<sub>10</sub> in plasma was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis [18].

### Immunohistochemistry and TUNEL staining

Immunohistochemistry was performed as described previously [2]. Oxidative damage markers (8-hydroxy-2'-deoxyguanosine [8-OHdG] and 4-hydroxy-2-hexenal [4-HHE]), and pro-inflammatory marker (ED-1) were detected in 4- $\mu$ m tissue sections by incubating the cells for 12 hours with specific antibodies against 8-OHdG (JaICA), 4-HHE

(JaICA, Shizuoka, Japan), and ED-1 (AbDSerotec, Oxford, UK) at 4°C. The TdT-mediated dUTP nick-end labeling (TUNEL) staining was performed and evaluated using the *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA, USA) as per the manufacturer's protocols. All data were analyzed using a color image analyzer (TDI Scope Eye version 3.6 for Windows, Seoul, Korea). Each section was counted in 20 different fields at 200 $\times$  magnification.

### Immunoblot analysis

Immunoblot analysis was performed as described previously [19,20]. Using tissue lysates from the renal cortex, E-cadherin (BD Biosciences, San Jose, CA, USA),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), collagen type IV, active caspase-3, BCL2-associated X (BAX), and  $\beta$ -actin were detected by incubating for 12 hours with specific antibodies against E-cadherin (BD Biosciences),  $\alpha$ -SMA (Sigma, St Louis, MO, USA), collagen type IV (Abcam, Cambridge, UK), active caspase-3 (Millipore), Bax (Delta Biolabs, Gilroy, CA, USA), and  $\beta$ -actin (Sigma) at 4°C.

### Measurement of interstitial fibrosis and glomerular injury in kidney

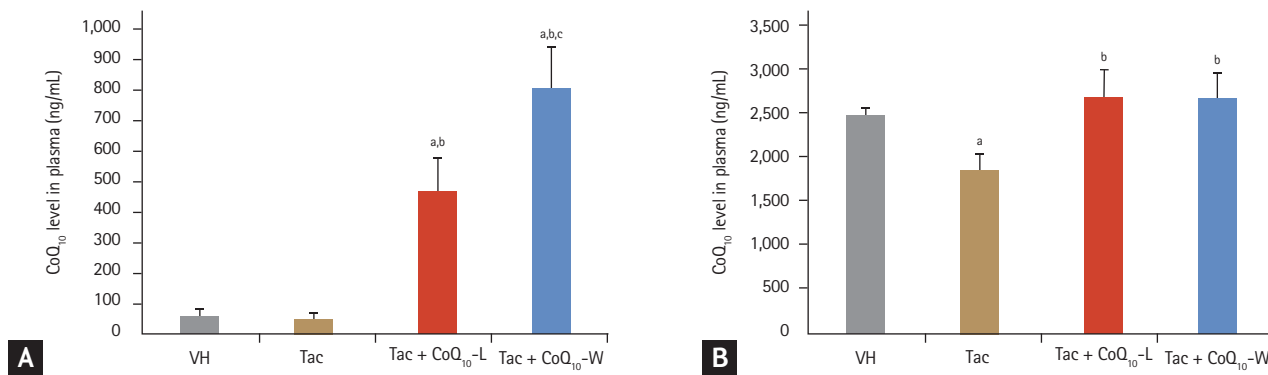
Histological assessment of tubule interstitial fibrosis (TIF) in trichrome-stained tissue sections was performed as described previously [21]. The extent of fibrosis was estimated by counting the percentage of injured area per field using a polygon program. A minimum of 20 fields per section was assessed and graded using color image analyzer (TDI Scope Eye version 3.0 for Windows, Olympus, Tokyo, Japan). Histopathological analyses were performed in randomly selected cortical fields of sections by a pathologist blinded to the identity of the treatment groups.

### 8-OHdG detection in serum and urine

All procedures strictly followed the protocol described in the enzyme linked immunosorbent assay (ELISA) kit (Cell Biolabs, San Diego, CA, USA).

### Transmission electron microscopy

Kidney cortex tissues were fixed in 2.5% glutaraldehyde (diluted in 0.1 M phosphate buffer), post-fixed with 1% Osmium (VIII) oxide (OsO<sub>4</sub>) and then embedded in Epon 812. Ultrathin sections were prepared using the embedded tissues, and the sections were stained with



**Figure 1.** Comparison of coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) concentration in plasma and kidney tissue between CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L in chronic tacrolimus (Tac) nephropathy. Concentration of CoQ<sub>10</sub> in plasma (A) and kidney tissue (B) was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Data are presented as the mean ± standard error (n = 9). One-way analysis of variance was used to analyze the data. VH, vehicle; Tac + CoQ<sub>10</sub>-L, co-treatment with lipid-soluble coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-W, co-treatment with water-soluble coenzyme Q<sub>10</sub>. <sup>a</sup>p < 0.05 vs. the VH group, <sup>b</sup>p < 0.05 vs. the Tac group, <sup>c</sup>p < 0.05 vs. the Tac + CoQ<sub>10</sub>-L group.

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 ×5,000 magnification. We counted mitochondria number per cell and measured mitochondrial area per cell in the scanned areas using an image analyzer (TDI Scope Eye version 3.6).

### Measurement of cell viability

Human kidney 2 (HK-2) cells were seeded into 96-well plates at a density of 2.5 × 10<sup>4</sup> cells/well. After pre-incubation in an incubator at 37°C for 24 hours, the culture medium was changed to serum-free medium containing Tac (50 µg/mL) and CoQ<sub>10</sub>-L or CoQ<sub>10</sub>-W (1, 10, 100 pg/mL; 1, 10, 100 ng/mL; and 1 µg/mL). Cell viability was assayed using a Cell Counting Kit-8 assay kit (Dojin Laboratories, Kumamoto, Japan) according to the manufacturer's protocol.

### Flow cytometry

Flow cytometry was performed to assess reactive oxygen species (ROS) production. HK-2 cells were seeded into 6-well plates at a density of 2.5 × 10<sup>5</sup> cells/well. After pre-incubation in an incubator at 37°C for 24 hours, the culture medium was changed to serum-free medium containing Tac (50 µg/mL) and CoQ<sub>10</sub>-L or CoQ<sub>10</sub>-W (1, 10, 100 pg/mL; 1, 10, 100 ng/mL; and 1 µg/mL). The evaluation method was performed as described previously [22].

### Statistics

All data are presented as the mean ± standard error (SE) of more than three independent experiments. Multiple comparisons between different groups were identified by one-way analysis of variance with Bonferroni *post hoc* tests using IBM SPSS Statistics version 24 (IBM, Armonk, NY, USA). Results with *p* values less than 0.05 were considered significant.

## RESULTS

### Comparison of CoQ<sub>10</sub> concentration between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in chronic Tac nephropathy

The concentration of CoQ<sub>10</sub> in plasma (Fig. 1A) in the VH and Tac groups was 57 ± 19 and 33 ± 10 ng/mL, respectively (*p* < 0.05). However, the CoQ<sub>10</sub> level in the Tac + CoQ<sub>10</sub>-W group was nearly twice as high as that in the Tac + CoQ<sub>10</sub>-L group (888 ± 101 ng/mL vs. 463 ± 91 ng/mL, respectively; *p* < 0.05 vs. Tac + CoQ<sub>10</sub>-L group). In the kidney tissue (Fig. 1B), the CoQ<sub>10</sub> content was lower in the Tac group than in the VH (1,837 ± 196 ng/mL vs. 2,481 ± 78 ng/mL, respectively; *p* < 0.05 vs. VH group). An increase in CoQ<sub>10</sub> level was observed in both Tac + CoQ<sub>10</sub>-L and Tac + CoQ<sub>10</sub>-W groups compared with that in the Tac group (2,665 ± 316 ng/mL in the Tac + CoQ<sub>10</sub>-L group and 2,629 ± 319 ng/mL in the Tac + CoQ<sub>10</sub>-W, *p* < 0.05 vs. Tac group) in the kidney. However, there was no significant difference between the two groups.



**Table 1. Basic parameters in each group**

Variable	VH	Tac	Tac + CoQ <sub>10</sub> -L	Tac + CoQ <sub>10</sub> -W
Body weight, g	67 ± 3	48 ± 5 <sup>a</sup>	62 ± 1 <sup>b</sup>	62 ± 2 <sup>b</sup>
Water intake, mL/day	16 ± 2	23 ± 1 <sup>a</sup>	18 ± 1 <sup>b</sup>	20 ± 2 <sup>b</sup>
Urine volume, mL/day	30 ± 2	49 ± 6 <sup>a</sup>	35 ± 1 <sup>b</sup>	39 ± 4 <sup>b</sup>
Scr, mg/dL	0.31 ± 0.03	0.43 ± 0.4 <sup>a</sup>	0.37 ± 0.03 <sup>a</sup>	0.30 ± 0.02 <sup>b,c</sup>
Tac conc., ng/mL	-	9 ± 4	9 ± 2	9 ± 3

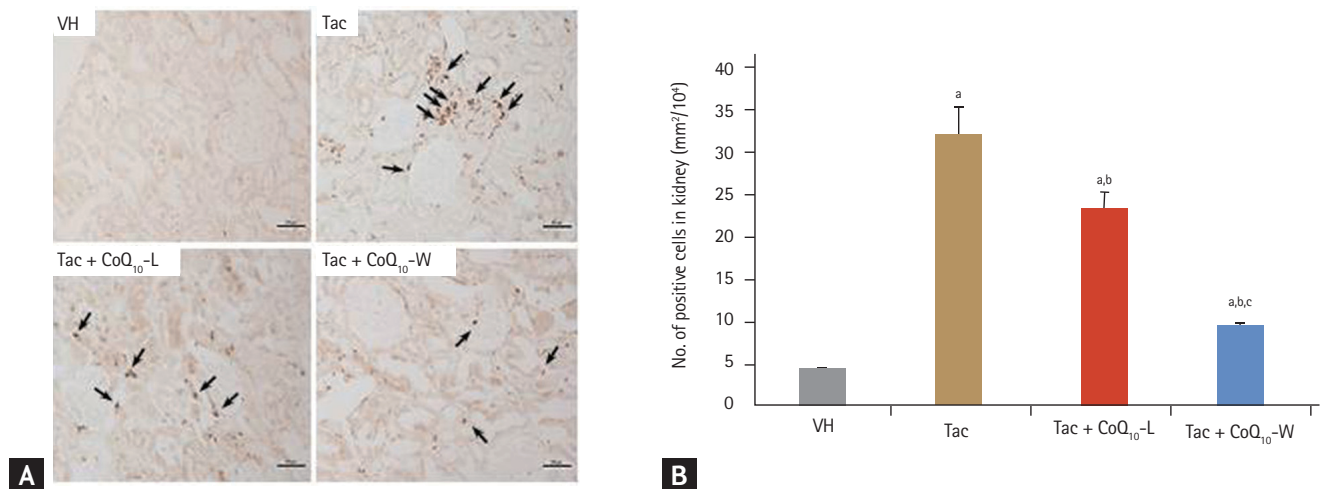
Values are presented as mean ± standard error (n = 9).

VH, vehicle; Tac, tacrolimus; CoQ<sub>10</sub>-L, lipid-soluble coenzyme q10; CoQ<sub>10</sub>-W, water-soluble CoQ<sub>10</sub>; Scr, serum creatinine; Tac conc., trough concentration of Tac.

<sup>a</sup>p < 0.05 vs. VH.

<sup>b</sup>p < 0.05 vs. Tac.

<sup>c</sup>p < 0.05 vs. Tac + CoQ<sub>10</sub>-L.



**Figure 2.** Comparison of anti-inflammatory effect between CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L in chronic tacrolimus (Tac) nephropathy. Representative photomicrographs of immunohistochemistry for ED-1 in experimental groups (A) and semi-quantitative analysis (B). Arrows indicate the ED-1 positive cells. Data are presented as the mean ± standard error (n = 9). Scale bar = 100 μm. One-way analysis of variance was used to analyze the data. VH, vehicle; CoQ<sub>10</sub>, coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-L, co-treatment with lipid-soluble coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-W, co-treatment with water-soluble coenzyme Q<sub>10</sub>. <sup>a</sup>p < 0.05 vs. the VH group, <sup>b</sup>p < 0.05 vs. the Tac group, <sup>c</sup>p < 0.05 vs. the Tac + CoQ<sub>10</sub>-L group.

### Comparison of renal function and basic parameters between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in chronic Tac nephropathy

Table 1 summarizes the characteristics of each group of rats after daily treatment with Tac with/without CoQ<sub>10</sub> for 4 weeks. Compared with the VH group, the Tac group had significantly changed body weight, water intake, and urine volume. Tac treatment alone resulted in higher levels of serum creatinine than that in VH-treated rats; however co-treatment with CoQ<sub>10</sub>-L or CoQ<sub>10</sub>-W along with Tac normalized kidney function.

### Comparison of anti-inflammatory effect between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in chronic Tac nephropathy

We investigated the infiltration of ED-1 positive cells in kidney tissue to evaluate the effect of CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W on Tac-induced inflammatory processes (Fig. 2). As shown in Fig. 2A, the number of ED-1-positive cells was decreased in the VH group. However, Tac treatment markedly increased these numbers, and this increase was markedly attenuated by treatment with CoQ<sub>10</sub>. Interestingly, addition of CoQ<sub>10</sub>-W showed a much reduced number of ED-1-positive cells compared to that with CoQ<sub>10</sub>-L during Tac treatment. Quantitative

analysis of Fig. 2A results demonstrate that there was a great contrast in the number of ED-1 positive cells between the VH and Tac groups ( $432 \pm 3 \text{ mm}^2/10^4$  vs.  $3 \pm 0.3 \text{ mm}^2/10^4$ , respectively;  $p < 0.05$  vs. VH group). However, there was a marked reduction in ED-1 positive cells in the Tac + CoQ<sub>10</sub>-W group compared with that in the Tac + CoQ<sub>10</sub>-L group ( $9.2 \pm 0.4 \text{ mm}^2/10^4$  vs.  $23.5 \pm 1.8 \text{ mm}^2/10^4$ , respectively;  $p < 0.05$  vs. Tac + CoQ<sub>10</sub>-L group).

### Comparison of anti-fibrotic effect between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in chronic Tac nephropathy

Fig. 3 shows that co-treatment with CoQ<sub>10</sub>-W or CoQ<sub>10</sub>-L had an anti-fibrotic effect in Tac-induced nephropathy. Fig. 3A shows that Tac treatment alone increased extensive tubule interstitial fibrosis compared with that in the VH group ( $16.3\% \pm 0.4\%$  vs.  $4.5\% \pm 0.1\%$ , respectively;  $p < 0.05$  vs. VH group). Concurrent treatment with CoQ<sub>10</sub> decreased the tubule interstitial fibrosis level compared to that with Tac treatment; however, CoQ<sub>10</sub>-W attenuated the tubule interstitial fibrosis level further than that by CoQ<sub>10</sub>-L ( $11\% \pm 0.4\%$  vs.  $13\% \pm 0.3\%$ , respectively;  $p < 0.05$  vs. Tac + CoQ<sub>10</sub>-L group). At the molecular level, the expression levels of E-cadherin were lower ( $62\% \pm 3\%$  vs.  $100\% \pm 0.1\%$ ,  $p < 0.05$  vs. VH group) and those of  $\alpha$ -SMA ( $130\% \pm 5.5\%$  vs.  $100\% \pm 0.1\%$ ,  $p < 0.05$  vs. VH group,  $p < 0.05$ ), collagen IV ( $231.9\% \pm 19.4\%$  vs.  $100\% \pm 0.1\%$ ,  $p < 0.05$  vs. VH group,  $p < 0.05$ ) after Tac treatment than in the VH group. These levels were restored by co-treatment with CoQ<sub>10</sub>-L or CoQ<sub>10</sub>-W. Especially, treatment with CoQ<sub>10</sub>-W significantly changed the E-cadherin ( $89\% \pm 2.8\%$  vs.  $75.5\% \pm 2.7\%$ ,  $p < 0.05$  vs. CoQ<sub>10</sub>-L group),  $\alpha$ -SMA ( $121\% \pm 11\%$  vs.  $148\% \pm 6\%$ ,  $p < 0.05$  vs. CoQ<sub>10</sub>-L group) and collagen IV ( $165.3\% \pm 6.4\%$  vs.  $181.6\% \pm 7.5\%$ ,  $p < 0.05$  vs. CoQ<sub>10</sub>-L group,  $p < 0.05$ ) levels compared to those by CoQ<sub>10</sub>-L (Fig. 3C–3E).

### Comparison of anti-oxidative effect between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in chronic Tac nephropathy

Fig. 4 shows the results for 8-OHdG in the kidney tissues and serum and 24-hour urinary excretion level. The expression of 8-OHdG was significantly higher in the Tac group than in the VH group ( $288 \pm 16 \text{ mm}^2/10^4$  vs.  $122 \pm 10 \text{ mm}^2/10^4$ , respectively;  $p < 0.05$  vs. VH group), and decreased by co-treatment with CoQ<sub>10</sub>-L ( $231 \pm 10 \text{ mm}^2/10^4$ ;  $p < 0.05$  vs. Tac group) or CoQ<sub>10</sub>-W ( $142 \pm 5 \text{ mm}^2/10^4$ ;  $p < 0.05$  vs. Tac group). Higher levels of 8-OHdG in se-

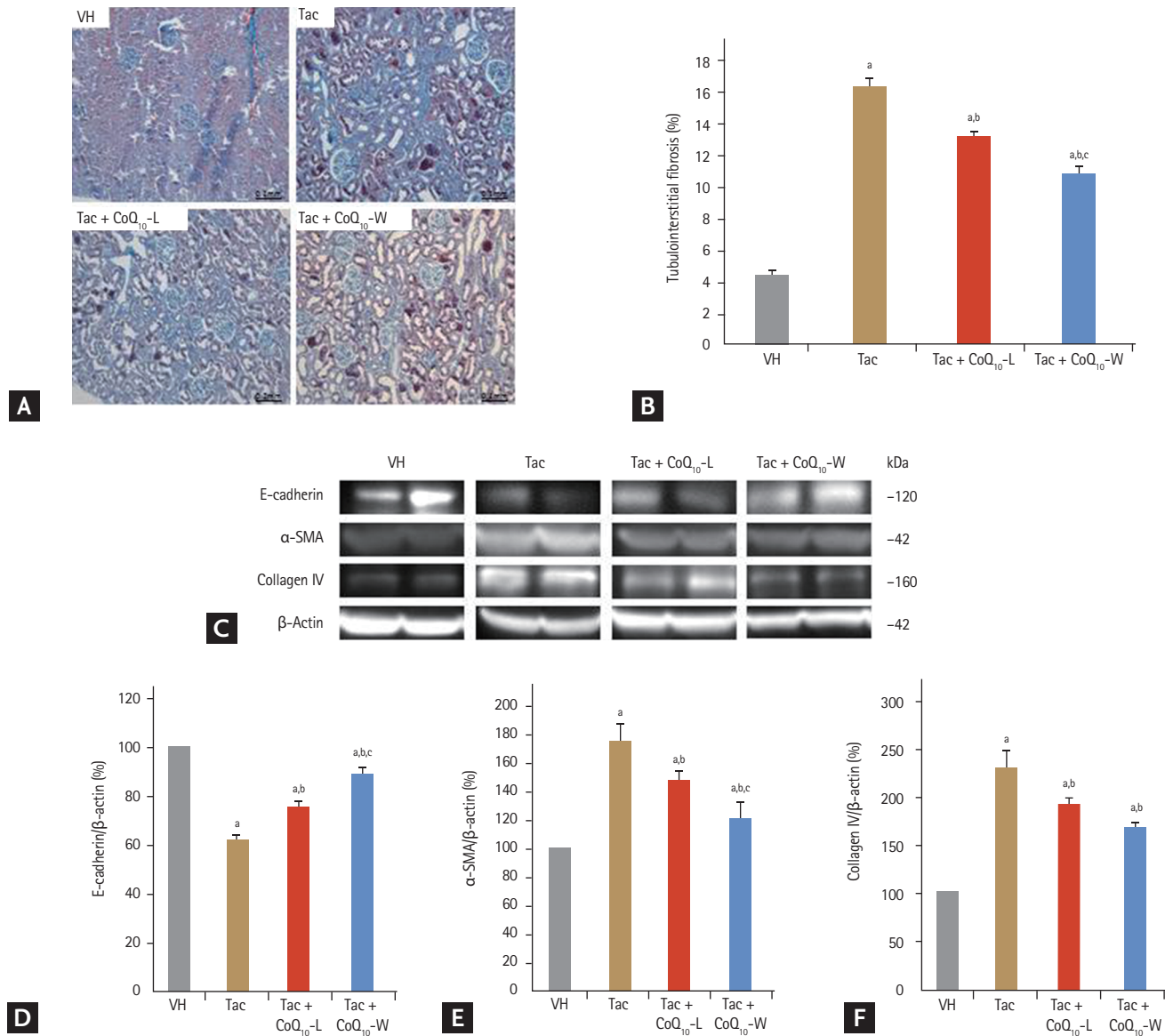
rum and urine were also detected in the Tac group than those in the VH group (serum,  $9.4 \pm 0.5 \text{ ng/mL}$  vs.  $7.8 \pm 0.5 \text{ ng/mL}$ ;  $p < 0.05$  vs. VH group; urine,  $90.3 \pm 12.1 \text{ ng/mL/day}$  vs.  $39.8 \pm 10.8 \text{ ng/mL/day}$ ;  $p < 0.05$  vs. VH group) however, significant changes were only observed in the CoQ<sub>10</sub>-L (serum,  $8.3 \pm 0.4 \text{ ng/mL}$ ; urine,  $59 \pm 8.6 \text{ ng/mL/day}$ ;  $p < 0.05$  vs. Tac group) and CoQ<sub>10</sub>-W (serum,  $7 \pm 0.4 \text{ ng/mL}$  vs. urine,  $32.4 \pm 13.7 \text{ ng/mL/day}$ ;  $p < 0.05$  vs. Tac group) groups (Fig. 4C and 4D). Tac + CoQ<sub>10</sub>-W reduced 8-OHdG expression further than that by Tac + CoQ<sub>10</sub>-L in the kidney tissue, serum, and 24-urine during treatment with Tac. Tac-induced 4-HHE expression also showed results consistent with those of 8-OHdG as shown in Fig. 5 (Tac + CoQ<sub>10</sub>-L,  $21.5 \pm 0.7 \text{ mm}^2/10^{10}$  vs. Tac + CoQ<sub>10</sub>-W,  $7.1 \pm 0.4 \text{ mm}^2/10^4$ ;  $p < 0.05$ ).

### Comparison of anti-apoptotic effect between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in chronic Tac nephropathy

We evaluated the effects of CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W on Tac-induced apoptosis using TUNEL assay in tissue sections (Fig. 6A and 6B). The maximum number of TUNEL-positive cells was observed in the Tac group and the minimum in the VH group ( $15.2 \pm 1.2$  vs.  $5 \pm 0.1$ , respectively;  $p < 0.05$  vs. VH group). As expected, the number of TUNEL-positive cells was reduced by co-treatment with CoQ<sub>10</sub>-L or CoQ<sub>10</sub>-W compared to that in the Tac group; however, Tac + CoQ<sub>10</sub>-W attenuated the number of TUNEL-positive cells much more than that by Tac + CoQ<sub>10</sub>-L ( $8.1 \pm 0.3 \text{ mm}^2/10^4$  vs.  $11.7 \pm 1.6 \text{ mm}^2/10^4$ , respectively;  $p < 0.05$  vs. Tac + CoQ<sub>10</sub>-L group). At the molecular level, the expression levels of active caspase-3 and BAX were higher with Tac treatment than in the VH group as shown in Fig. 6C–6E. These levels were restored by co-treatment with CoQ<sub>10</sub>-L or CoQ<sub>10</sub>-W. Especially, CoQ<sub>10</sub>-W significantly reduced the expression of these proteins compared to that by CoQ<sub>10</sub>-L (active caspase-3,  $112\% \pm 6\%$  vs.  $137\% \pm 9\%$ ,  $p < 0.05$  vs. Tac + CoQ<sub>10</sub>-L group; BAX,  $114\% \pm 7\%$  vs.  $158\% \pm 17\%$ ,  $p < 0.05$  vs. Tac + CoQ<sub>10</sub>-L group).

### Comparison of mitochondrial structure between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in chronic Tac nephropathy

We evaluated the effect of CoQ<sub>10</sub> administration on Tac-induced mitochondrial ultrastructural changes in kidney tissue using electron microscopy to assess the size and the number of mitochondria (Fig. 7). Tac treat-



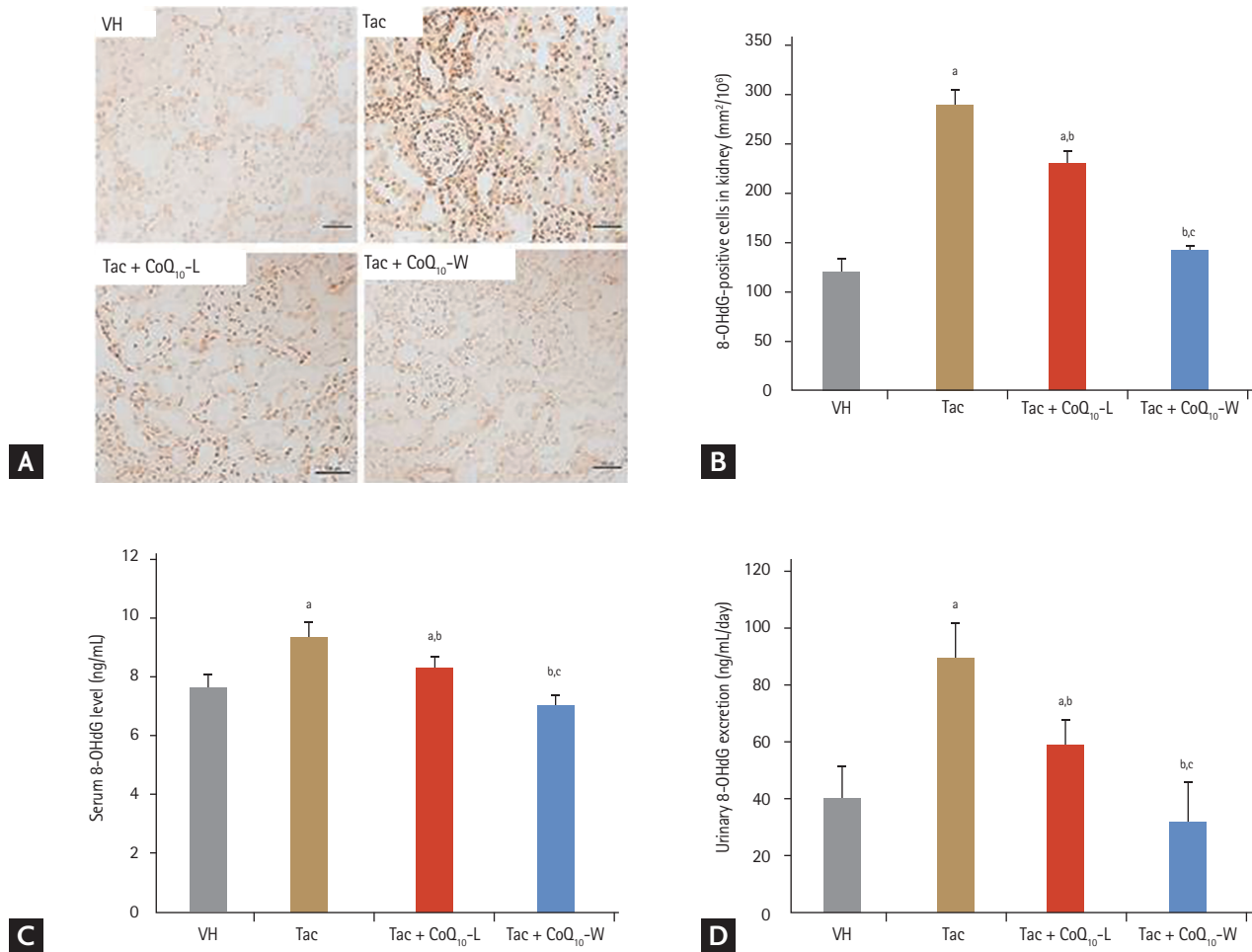
**Figure 3.** Comparison of anti-fibrotic effect between CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L in chronic tacrolimus (Tac) nephropathy. Representative photomicrographs of immunohistochemistry for trichrome in experimental groups (A) and semi-quantitative analysis (B). Immunoblot analysis of E-cadherin,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and collagen IV (C, D, E, F). Data are presented as the mean  $\pm$  standard error (n = 9). Scale bar = 100  $\mu$ m. The relative optical densities of bands in each lane were normalized to each  $\beta$ -actin band from the same gel. One-way analysis of variance was used to analyze the data. VH, vehicle; CoQ<sub>10</sub>, coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-L, co-treatment with lipid-soluble coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-W, co-treatment with water-soluble coenzyme Q<sub>10</sub>. <sup>a</sup>*p* < 0.05 vs. the VH group, <sup>b</sup>*p* < 0.05 vs. the Tac group, <sup>c</sup>*p* < 0.05 vs. the Tac + CoQ<sub>10</sub>-L group.

ment decreased both average mitochondrial size ( $0.35 \pm 0.01 \mu\text{m}^2$  vs.  $0.57 \pm 0.02 \mu\text{m}^2$ , *p* < 0.05 vs. VH group) and number ( $0.46 \pm 0.02$  vs.  $0.79 \pm 0.04$ , *p* < 0.05 vs. VH group). However, co-treatment with CoQ<sub>10</sub>-L (size,  $0.46 \pm 0.02 \mu\text{m}^2$ ; number,  $0.7 \pm 0.04 \mu\text{m}^2$ ; *p* < 0.05 vs. Tac group) and CoQ<sub>10</sub>-W (size,  $0.49 \pm 0.01 \mu\text{m}^2$ ; number,  $0.73 \pm 0.09 \mu\text{m}^2$ ;

*p* < 0.05 vs. Tac group) restored these changes compared with those by Tac alone (Fig. 7B and 7C).

#### Comparison of cell viability between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in Tac-induced HK-2 cell injury

Fig. 8 demonstrates the protective effect of CoQ<sub>10</sub>-L and



**Figure 4.** Comparison of the expression of 8-hydroxy-2'-deoxyguanosine (8-OHdG) between CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L in chronic tacrolimus (Tac) nephropathy. Representative photomicrographs of immunohistochemistry for 8-OHdG (A, B) using tissue sections from rats. Quantification of 8-OHdG level using the enzyme linked immunosorbent assay (ELISA) kit (C, D) using serum and urine from rats. Data are presented as the mean ± standard error (n = 9). Scale bar = 100 μm. One-way analysis of variance was used to analyze the data. VH, vehicle; CoQ<sub>10</sub>, coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-L, co-treatment with lipid-soluble coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-W, co-treatment with water-soluble coenzyme Q<sub>10</sub>. <sup>a</sup>*p* < 0.05 vs. the VH group, <sup>b</sup>*p* < 0.05 vs. the Tac group, <sup>c</sup>*p* < 0.05 vs. the Tac + CoQ<sub>10</sub>-L group.

CoQ<sub>10</sub>-W against cell injury during Tac treatment in the renal proximal tubule cell line, HK-2 cells. The different doses of Tac + CoQ<sub>10</sub>-L and Tac + CoQ<sub>10</sub>-W increased the cell viability of HK-2 cells by approximately 2.8 times compared with that in the Tac group. However, there was no significant difference in cell viability between the Tac + CoQ<sub>10</sub>-L and the Tac + CoQ<sub>10</sub>-W groups at the indicated doses.

**Comparison of ROS production between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in Tac-induced HK-2 cell injury**

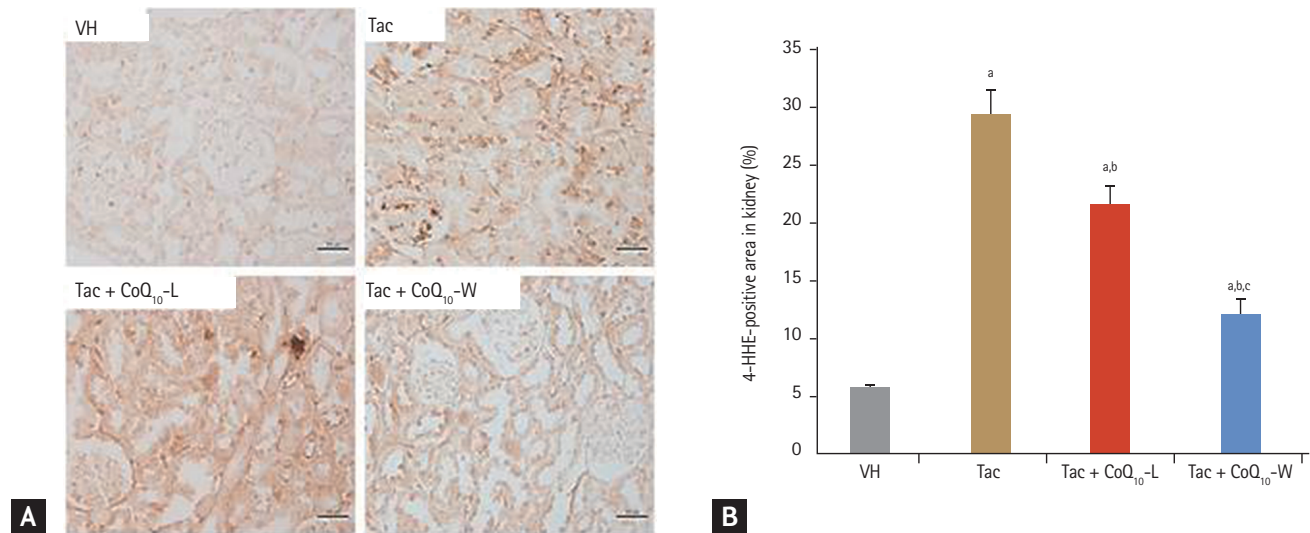
Co-treatment with CoQ<sub>10</sub>-L or CoQ<sub>10</sub>-W reduced the

Tac-induced MitoSOX Red fluorescence level, which is a marker for mitochondrial ROS; however, as shown in Fig. 9, no difference was observed in the protective level between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W.

**DISCUSSION**

The hypothesis of our study was that improvement in bioavailability of CoQ<sub>10</sub> may provide better protection against Tac-induced renal injury, and we compared the efficacy between CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L in an experi-





**Figure 5.** Comparison of the expression of 4-hydroxy-2-hexenal (4-HHE) between CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L in chronic tacrolimus (Tac) nephropathy. Representative photomicrographs of immunohistochemistry for 4-HHE in experimental groups (A) and semi-quantitative analysis (B). Data are presented as the mean  $\pm$  standard error (n = 9). Scale bar = 100  $\mu$ m. One-way analysis of variance was used to analyze the data. VH, vehicle; CoQ<sub>10</sub>, coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-L, co-treatment with lipid-soluble coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-W, co-treatment with water-soluble coenzyme Q<sub>10</sub>. <sup>a</sup>*p* < 0.05 vs. the VH group, <sup>b</sup>*p* < 0.05 vs. the Tac group, <sup>c</sup>*p* < 0.05 vs. the Tac + CoQ<sub>10</sub>-L group.

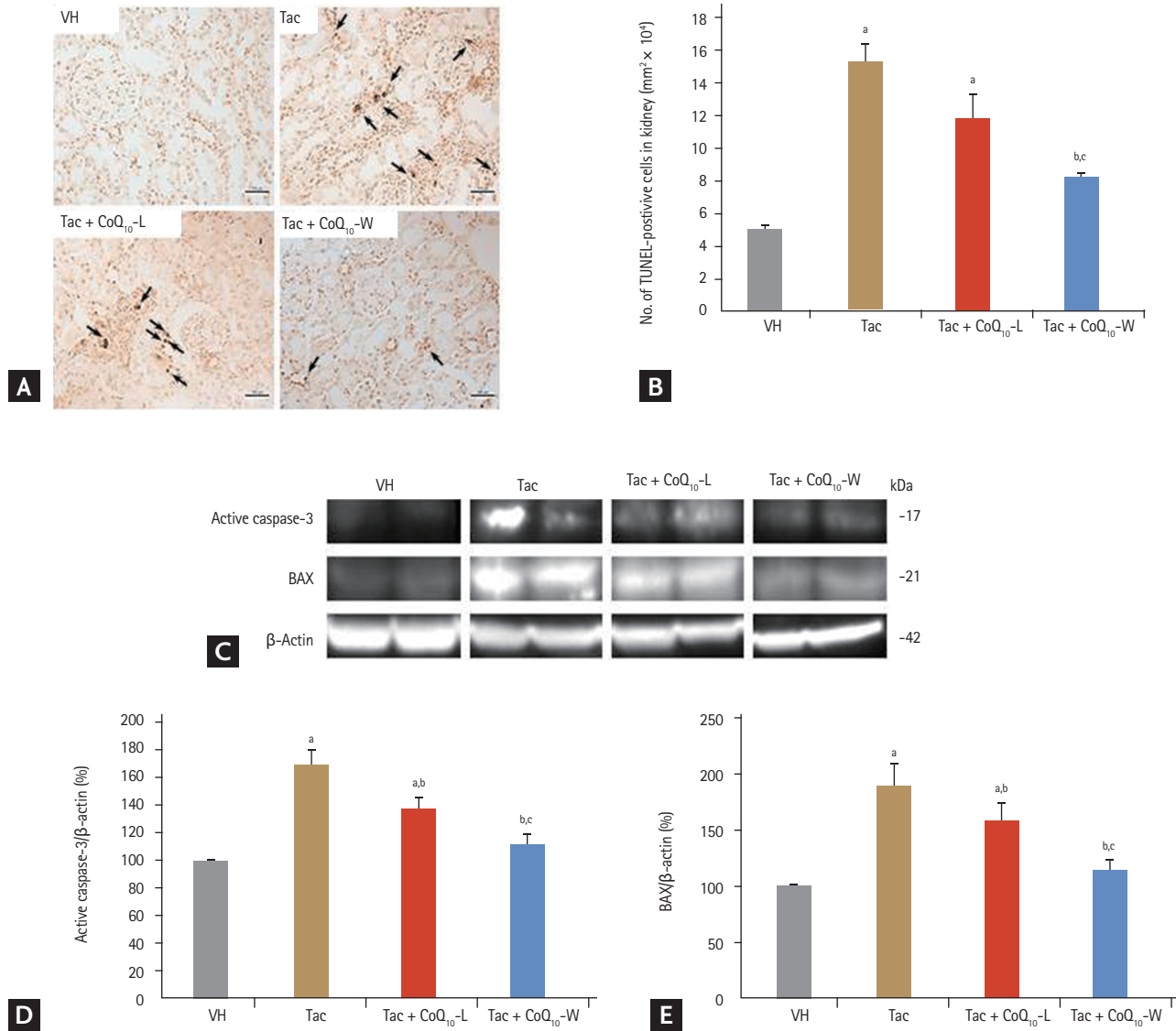
mental model of chronic Tac nephropathy. The results of the study demonstrate that CoQ<sub>10</sub>-W increased renal function and histology better than CoQ<sub>10</sub>-L. In addition, addition of CoQ<sub>10</sub>-W reduced Tac-induced inflammation, oxidative stress, and apoptosis in the renal tissues more than that by CoQ<sub>10</sub>-L addition. These findings suggest that improved bioavailability of CoQ<sub>10</sub>-W may contribute to reducing Tac-induced nephropathy owing to its action as an anti-oxidative agent.

It is well known that oxidative stress and apoptosis are common mechanisms of Tac-induced nephropathy. To reveal the improvement in renal injury by CoQ<sub>10</sub>-W, we compared the antioxidant and anti-apoptotic effects between CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L in Tac-induced nephropathy. The results of our study clearly demonstrate that CoQ<sub>10</sub>-W reduced both Tac-induced markers of oxidative stress (8-OHdG in tissue, serum, and urine; 4-HHE in tissue) and Tac-induced apoptosis to a greater extent than CoQ<sub>10</sub>-L. These findings confirm that CoQ<sub>10</sub>-W has better antioxidant and anti-apoptotic effects than CoQ<sub>10</sub>-L on Tac-induced renal injury, and this may explain the better protection of CoQ<sub>10</sub>-W than CoQ<sub>10</sub>-L.

We previously reported the efficacy of CoQ<sub>10</sub>-W in Tac-induced diabetes mellitus [23], and this study ex-

tended the evaluation of this effect to Tac-induced renal injury. The results of our study clearly demonstrate that CoQ<sub>10</sub>-W significantly improved renal function and histopathological parameters compared to that with CoQ<sub>10</sub>-L in a rat model of Tac-induced renal injury. This finding suggests that CoQ<sub>10</sub>-W is a more effective antioxidant than CoQ<sub>10</sub>-L, and this is related to the increased bioavailability of CoQ<sub>10</sub>-W as shown in plasma CoQ<sub>10</sub> levels (Fig. 1A). However, kidney tissue CoQ<sub>10</sub> levels were not significantly different in the two groups. Thus, the improved renal function and histopathology in the CoQ<sub>10</sub>-W group compared with that in the CoQ<sub>10</sub>-L group seems to be related to a systemic antioxidant effect.

Oxidative stress is closely related to the quantity and quality of mitochondria [24,25]. High-quality mitochondria protect cells against oxidative stress and prevent apoptosis. In our study, CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L both increased the number and area of mitochondria in cells in comparison with that seen in the Tac group, but there was no significant difference. Therefore, while we cannot clearly demonstrate the reason, we speculate that the subcellular effects of CoQ<sub>10</sub>-W are not significantly different from those of CoQ<sub>10</sub>-L owing to a similar

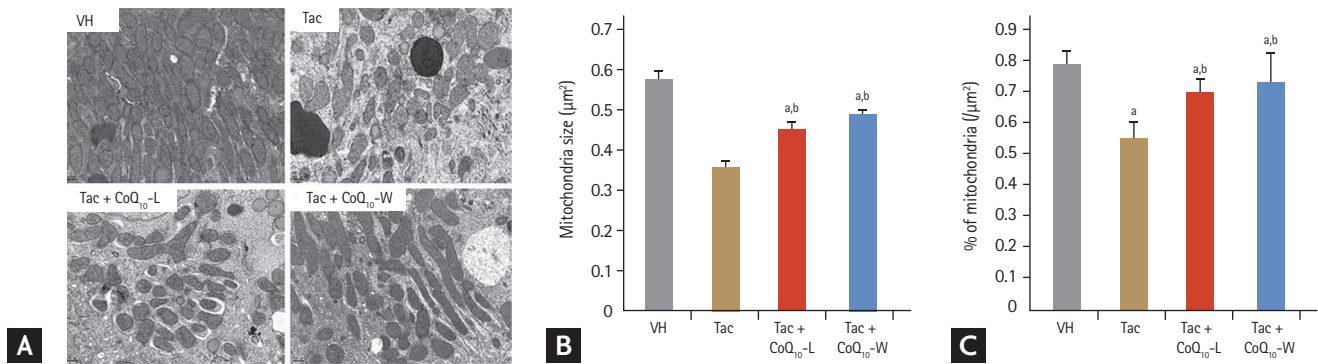


**Figure 6.** Comparison of anti-apoptotic effect between CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L in chronic tacrolimus (Tac) nephropathy. Representative photomicrographs of immunohistochemistry for TdT-mediated dUTP nick-end labeling (TUNEL) in experimental groups (A) and semi-quantitative analysis (B). Arrows indicate the TUNEL positive cells. Immunoblot analysis of active caspase-3 and BCL2-associated X (BAX) (C, D, E). Data are presented as the mean ± standard error (n = 9). Scale bar = 100 μm. The relative optical densities of bands in each lane were normalized to each β-actin band from the same gel. One-way analysis of variance was used to analyze the data. Relative active caspase-3 and BAX are referenced to β-actin. VH, vehicle; CoQ<sub>10</sub>, coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-L, co-treatment with lipid-soluble coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-W, co-treatment with water-soluble coenzyme Q<sub>10</sub>. <sup>a</sup>*p* < 0.05 vs. the VH group, <sup>b</sup>*p* < 0.05 vs. the Tac group, <sup>c</sup>*p* < 0.05 vs. the Tac + CoQ<sub>10</sub>-L group.

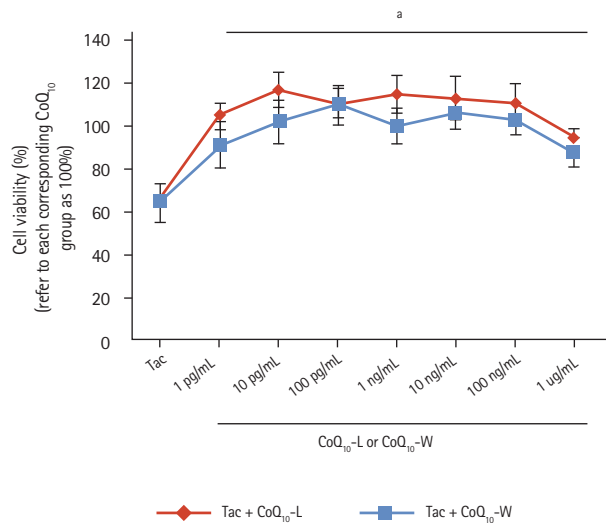
CoQ<sub>10</sub> renal content level as shown in Fig. 1B.

According to the previous report [5], the CoQ<sub>10</sub> level responded in a CoQ<sub>10</sub> dose dependent manner. Therefore, increasing doses of conventional lipid soluble-CoQ<sub>10</sub> has been thought to be better than newly developing formulation of CoQ<sub>10</sub>. However, a study reported that in

rats, only 3% of orally administered lipid soluble CoQ<sub>10</sub> was absorbed [8]. Due to its lack of solubility, therapeutic application of lipid soluble CoQ<sub>10</sub> are limited by its poor bio-availability. In particular, water-soluble CoQ<sub>10</sub> formulation not lipid-soluble CoQ<sub>10</sub> may increase the mitochondrial content of CoQ<sub>10</sub> in order to improve their



**Figure 7.** Comparison of mitochondrial structure between CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L in chronic tacrolimus (Tac) nephropathy. Representative images using transmission electron microscopy (A) and quantification (B, C) of electron microscopy. Scale bar = 500 nm. Data are presented as the mean ± standard error (n = 9). One-way analysis of variance was used to analyze the data. VH, vehicle; CoQ<sub>10</sub>, coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-L, co-treatment with lipid-soluble coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-W, co-treatment with water-soluble coenzyme Q<sub>10</sub>. <sup>a</sup>*p* < 0.05 vs. the VH group, <sup>b</sup>*p* < 0.05 vs. the Tac group.



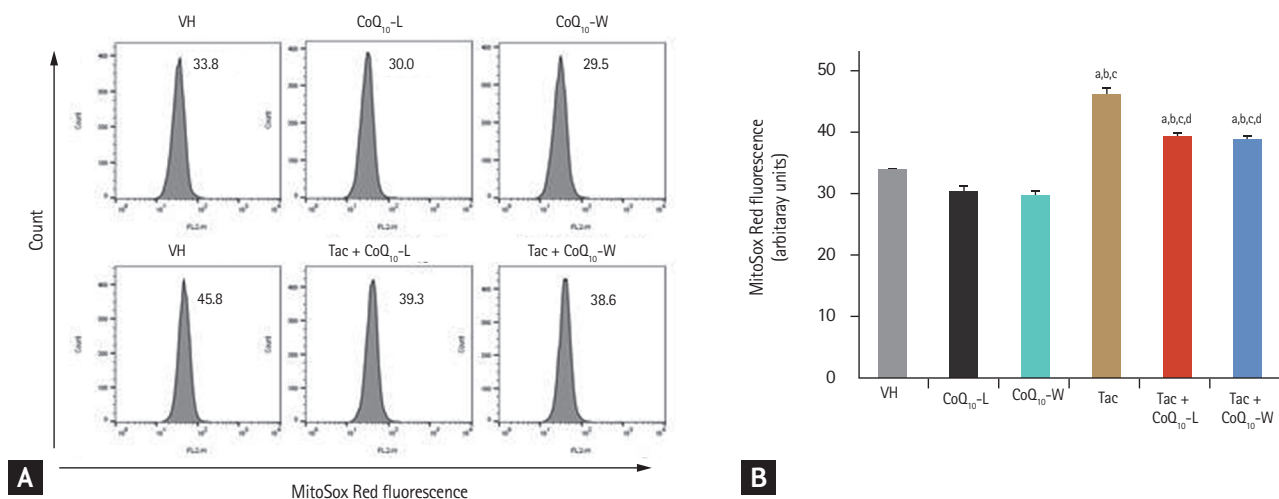
**Figure 8.** Comparison of cell viability between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in tacrolimus (Tac)-induced human kidney 2 (HK-2) cell injury. Cell viability was evaluated by HK-2 assay. Data are presented as the mean ± standard error (n = 7). One-way analysis of variance was used to analyze the data. CoQ<sub>10</sub>, coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-L, co-treatment with lipid-soluble coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-W, co-treatment with water-soluble coenzyme Q<sub>10</sub>. <sup>a</sup>*p* < 0.05 vs. the Tac group.

bioenergetics parameter [5]. Therefore, development of water-soluble formulation of CoQ<sub>10</sub> has potentially benefit on disorders related to mitochondrial dysfunctions.

Our study has some limitations. First, there was no difference between CoQ<sub>10</sub>-W and CoQ<sub>10</sub>-L of the CoQ<sub>10</sub> lev-

el in the kidney tissue. *In vitro* experiments also showed similar time and dose-response protective effect in a cell viability and mitochondrial ROS level in the cellular level between these two groups. Therefore, follow-up study is required to improve the action of CoQ<sub>10</sub>-W at the cellular level. Second, CoQ<sub>10</sub> is known as the endogenous substances that follows the circadian rhythm [26]. In this study, we examined the CoQ<sub>10</sub> levels at the end of the experimental period. For more accurate estimate of CoQ<sub>10</sub> level, pharmacokinetic and pharma co-dynamic studies are needed to determine whether CoQ<sub>10</sub>-W has better bioavailability than CoQ<sub>10</sub>-L. Third, CoQ<sub>10</sub>-W consist of EPA for consistent nano-emulsion. Therefore, there is a possibility that the anti-inflammatory effect of EPA may be overlapped with that of CoQ<sub>10</sub>-W. Regarding this, we previously tested the anti-inflammatory effect of EPA in a rheumatoid arthritis animal model, and found that EPA in nano-emulsion has not affected the anti-inflammatory effect of CoQ<sub>10</sub> (data not shown). Thus, the anti-inflammatory effect of EPA in nano-emulsion may be ruled out in the present study.

In summary, the improved CoQ<sub>10</sub> bioavailability using CoQ<sub>10</sub>-W may provide more benefit than conventional CoQ<sub>10</sub> in Tac-induced renal injury. Thus, CoQ<sub>10</sub>-W supplementation may be beneficial in patient prescribed Tac following renal transplantation.



**Figure 9.** Comparison of reactive oxygen species (ROS) production between CoQ<sub>10</sub>-L and CoQ<sub>10</sub>-W in tacrolimus (Tac)-induced human kidney 2 (HK-2) cell injury. Intra cellular ROS production using a flow cytometer. MitoSOX Red was used as a probe to evaluate (A) intracellular ROS alterations in mitochondria (B) quantitative analysis of ROS production. Data are presented as the mean ± standard error (n = 9). One-way analysis of variance was used to analyze the data. VH, vehicle; CoQ<sub>10</sub>, coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-L, co-treatment with lipid-soluble coenzyme Q<sub>10</sub>; Tac + CoQ<sub>10</sub>-W, co-treatment with water-soluble coenzyme Q<sub>10</sub>. <sup>a</sup>p < 0.05 vs. the VH group, <sup>b</sup>p < 0.05 vs. the CoQ<sub>10</sub>-L group, <sup>c</sup>p < 0.05 vs. the CoQ<sub>10</sub>-W group, <sup>d</sup>p < 0.05 vs. the Tac group.

### KEY MESSAGE

1. Water-soluble coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) improves renal function and histopathology compared to that with conventional lipid-soluble CoQ<sub>10</sub> in chronic tacrolimus (Tac) nephropathy.
2. Water-soluble CoQ<sub>10</sub> reduces oxidative stress more effectively than lipid-soluble CoQ<sub>10</sub> in chronic Tac nephropathy.
3. Treatment with a combination of water-soluble CoQ<sub>10</sub> and Tac provides effective protection against Tac-induced renal injury.

### Conflict of interest

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

### Acknowledgments

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