Beneficial effects of dapagliflozin and pioglitazone combination in diabetic nephropathy

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Beneficial effects of dapagliflozin and pioglitazone combination in diabetic nephropathy

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The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Doctor of philosophy

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December 2017

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December 2017

ACKNOWLEDGEMENTS

I would like to express my special appreciation and thanks to my advisor Professor Dr. Bong-Soo Cha, who has been a tremendous mentor for me. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. Your advice on both research as well as on my career have been priceless. I would like to thank professor Sahng Wook Park, professor Hyeon Joo Jeong, professor Eun-Jung Rhee, and professor Yong-ho Lee for serving as my committee members even at hardship. It was an enjoyable moment to defense for your brilliant comments and suggestions. I would especially like to thank my colleagues during fellowship at Yonsei University, Severance Hospital. All of you have been there to support me when I experimented and collected data for my Ph.D. thesis.

A special thanks to my family. Words cannot express how grateful I am to my mother, father, grandmother, brother for all of the sacrifices that you've made on my behalf. Your prayers for me were what sustained me thus far. I would also like to thank all of my friends who supported me in writing, and encouraged me to strive towards my goal.

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ABSTRACT

Benefical effect of dapagliflozin and pioglitazone combination in diabetic nephropathy

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The therapeutic efficacy of combination therapy on diabetic nephropathy has not been investigated although dapagliflozin and pioglitazone have glucose-lowering and anti-inflammatory effects. In this study, 9-week-old male *db/db* mice were randomly assigned to 4 groups and administrated with (1) vehicle, (2) dapagliflozin, (3) pioglitazone, or (4) dapagliflozin and pioglitazone combination. For *in vitro* evaluation, human proximal tubule (HK-2) cells were treated with glucose or palmitate acid in the presence or absence of dapagliflozin or pioglitazone. The expression of proteins related to inflammation and renin-angiotensin system, and survival of HK-2 cells and mice kidneys were examined. Glomerular tuft area and mesangial expansion of the kidney were more reduced in the combination group compared to control and single therapy groups. Podocyte foot process width and glomerular basement membrane thickness decreased regardless of treatment, while the combination group showed the slowest renal hypertrophy progression ($p < 0.05$). In addition, the combination treatment decreased MCP-1, type I and IV collagen expression in the renal cortex. *In vitro* studies, only the

combination treatment decreased the expression of angiotensinogen, IL-6, and TGF-β while it enhanced HK-2 cell survival (all $p < 0.05$). In conclusion, dapagliflozin and pioglitazone preserved renal function in *db/db* mice, and combination therapy showed the greatest benefit. These findings suggest that the combination therapy of dapagliflozin with pioglitazone is more effective than the single therapy for preventing the progression of nephropathy in patients with type 2 diabetes.

Key words: sodium glucose co-transporter 2 inhibitor; thiazolidinedione; diabetic nephropathy; type 2 diabetes

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I. INTRODUCTION

Diabetic nephropathy is the leading cause of end-stage renal disease[1,2] and difficulties of restoring impaired kidney function emphasize the importance of diabetic nephropathy management.[3]

Sodium glucose co-transporter 2 (SGLT2) inhibitors block glucose reabsorption in the proximal tubules, and consequently stimulate glucose excretion in the urine.[4] Dapagliflozin is a highly selective and first-in-class SGLT2 inhibitor, which has many favorable effects on glucose lowering and body weight loss in clinical studies.[5,6] The result of preclinical and animal studies demonstrated other beneficial effects of this SGLT2 inhibitor such as improved glucose homeostasis,[7] preserved pancreatic islet cell function,[8,9] enhanced muscle insulin sensitivity,[10] and attenuated hepatic steatosis.[11]

Pioglitazone is in the class of thiazolidinedione (TZD), which plays essential roles in improving glucose tolerance and insulin sensitivity.[12] Although TZD has a protective effect against cardiovascular disease and inflammation,[13,14] this class of drug induces fluid retention and edema, and aggravates congestive heart failure because of increased sodium re-uptake.[15]

Because of the sodium excreting effect of dapagliflozin, it is plausible that dapagliflozin can prevent the peripheral edema that may be induced by pioglitazone treatment. Reducing excess glucose combined with improving insulin sensitivity could be an ideal combination for obese patients with type 2

diabetes (T2D). However, there is limited information on the combination therapy of SGLT2 inhibitor and TZD in diabetic nephropathy. We hypothesized that the combination therapy could have a synergistic effect or at least have an additive effect on preventing diabetic nephropathy in type 2 diabetes model. Therefore, the aim of the present study was to investigate the therapeutic effect of combination therapy in an animal model to support the experimental rationale for the combination therapy of pioglitazone and dapagliflozin.

II. MATERIALS AND METHODS

1. Experimental animals and study design

Eight-week-old male *db/db* mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). After 1 week of acclimatization, mice were divided into four groups: (1) vehicle control (p*hosphate-buffered saline* [*PBS; Amresco, Solon, OH, USA] solution)* (n = 5), (2) 30 mg/kg/day pioglitazone (n = 8), (3) 2 mg/kg/day dapagliflozin (n = 8), or (4) a combination of 2 mg/kg/day dapagliflozin plus 30 mg/kg/day pioglitazone (n = 7). Vehicle or drugs were administrated daily by oral gavage for 9 weeks. All animal studies were approved by the Animal Care and Use Committee of the Yonsei University College of Medicine.

2. Biochemical measurements

Blood samples for random glucose measurements were obtained via tail tip vein and glucose concentrations were determined with a glucose analyzer (AGM-4100; Allmedicus, Anyang, Korea). On week 8, spot urine was obtained as previously explained,[16] and stored at -80˚C for analysis. Urinary creatinine was determined using an autoanalyzer (Molecular Devices, Sunnyvale, CA, USA) and urinary albumin concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, Cambridge, UK) according to the manufacturer's protocol. At week

9, an oral glucose tolerance test was performed following a 6-hr fast,[17] and blood samples were taken via tail prior to (0 min) and following an oral glucose bolus (1 g/kg) at 30, 60, 90, and 120 min to measure plasma glucose concentration. At the end of treatment, general anesthesia was induced via inhalation of 5% isoflurane. Blood samples were obtained by left ventricular puncture and stored at -70˚C for subsequent analyses, were centrifuged at 5000× *g* for 15 min at 4˚C. Plasma concentrations of triglycerides (TG; BioVision, Milpitas, CA, USA) and free fatty acid (FFA; BioAssay Systems, Hayward, CA, USA) were measured using a colorimetric method according to the manufacturer's protocols.

3. Tissue collection and histological analysis

Paraffin-embedded kidney tissues were cut into 4 μm thick sections and stained with Hematoxylin and Eosin (H&E), Periodic Acid-Schiff (PAS), and Masson's trichrome stain. All tissue sections were examined using a BX40 microscope (OL-BX40, Olympus, Tokyo, Japan). Mesangial expansion and glomerular hypertrophy were assessed in a minimum of 15 glomeruli per mouse kidney. The tissue sections were magnified at ×400 and the diameter of the glomerular tuft and PAS-positive areas were quantified from the glomerulus cut in a plane along the vascular pole. For transmission electron microscopic analysis, kidney cortical samples were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4), followed by 1% osmium tetroxide. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation under a JEM 1011CX electron microscope (JEOL, USA, Inc.). Histological images were analyzed using ImageJ software (NIH Image, Bethesda, MD, USA) for quantifying mesangial expansion and glomerular hypertrophy.

4. *In situ* apoptosis detection

To investigate the apoptosis of kidney tubule, Terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-dUTP nick end labeling (TUNEL) was performed on fixed tissue sections using a commercially available kit (TACS® 2TdT DAB kit, Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Twenty randomly selected areas per mouse kidney were evaluated under high power magnification (×400).

5. Real-time PCR

The kidney RNA was prepared using Trizol reagent (Thermo Fisher, Grand Island, NY, USA) according to the manufacturer's instructions. Reverse transcription was performed using the high capacity complementary DNA transcription kit (Applied Biosystems, Foster City, CA, USA) by real time polymerase chain reaction (RT-PCR) using the SYBR Green Master Mix (Thermo Fisher, Grand Island, NY, USA). Expression of transforming growth factor (TGF)-β, monocyte chemoattractant protein (MCP)-1, type I and type IV collagens, renin, interleukin (IL)-6, and angiotensinogen (AGT) was normalized to the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 1. Primer sequences used for RT-PCR

Human primers	Sequences (5'→3')	GenBank reference sequences
β-actin	F-GGACTTCGAGCAAGAGATGG	NM_001101.4
	R-AGCACTGTGTTGGCGTACAG	→NP_001092.1
AGT	F-AAAGCAGCCGTTTCTCCTTG	NM_001101.4
	R-TTCACAAACAAGCTGGTCGG	→NP_001092.1
MCP-1	F-CCCCAGTCACCTGCTGTTAT	NM_002982.3
	R-TGGAATCCTGAACCCACTTC	→NP_002973.1
Renin	F-TCGTCTTTGACACTGGTTCGTCCA	NM_000537.3
	R-AGCCACTGACTGTCCCTGTTGAAT	→NP_000528.1
TGF-β	F-GGGACTATCCACCTGCAAGA	NM_000660.6
	R-CCTCCTTGGCGTAGTAGTCG	→NP_000651.3
IL-6	F-CCAGCTATGAACTCCTTCTC	NM_000600.4

	R-GCTTGTTCCTCACATCTCTC	→NP_000591.1
Mouse primers	Sequences (5'→3')	GenBank reference sequences
Gapdh	F-TGCCTCCTGCACCACCAACT	NP_001256799.2
	r- TGCCTCCTGCACCACCAACT	→NP_001243728
Agt	F-CCTCCCGACTAGATGGACAC	NM_007428.3
	r-AAATCCAGAGAGCGTGGGAA	→NP_031454.3
Mcp-1	F- TTAAAAACCTGGATCGGAACCAA	NM_011333.3
	r- GCATTAGCTTCAGATTTACGGGT	→NP_035463.1
Renin	F- CCTCTACCTTGCTTGTGGGA	NM_031192.3
	r- ATGCCTAGAACCCCGTCAAA	→NP_112469.1
Tgf-β	F-TGACGTCACTGGAGTTGTACGG	NM_021578.2
	r- GGTTCATGTCATGGATGGTGC	→NP_067589.1

RT-PCR, real time polymerase chain reaction; AGT, angiotensinogen; MCP-1, monocyte chemotactic protein-1; TGF-β, transforming growth factor beta 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

6. Cell culture

An immortalized human proximal tubule epithelial cell, HK-2 was maintained in Dulbeco's Modified Eagle's Media (DMEM) containing 25 mM D-glucose supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) (Thermo Fisher, Grand Island, NY, USA).[18] When cell confluency reached to 80% confluence, cells were exposed to (1) 5.5 mM glucose, (2) 50 mM glucose, (3) 50 mM glucose plus 0.3 mM palmitic acid (Sigma-Aldrich, Saint Louis, MO, USA), (4) 10 μM pioglitazone plus (3) medium, (5) 10 μM dapagliflozin plus (3) medium, or (6) 10 μM pioglitazone and 10 μM dapagliflozin co-treatment in (3) medium, for 24 hr then harvested.

7. Cell viability assay

HK-2 cells were seeded into in 96-well plates and incubated overnight to allow the cells to adhere and, were exposed to same manner to the condition used for cell culture study. The cells were then incubated with WST-8 (Dojindo Laboratories, Kumamoto, Kumamoto, Japan) solution at 37˚C for 1 hr and the

absorbance at 450 nm was measured using a microplate reader (VersaMax ELISA Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). The cell viability index was calculated as experimental value over denoted as percentage of obtained from control-treated cell.

8. Western blot

Membrane and cytoplasmic proteins were extracted from cultured HK-2 cell using the Mem-PER Plus Membrane Protein Extraction Kit (Pierce Biotechnology, Rockford, IL, USA) and measured suing the bicinchoninic acid assay (Pierce Biotechnology) according to the manufacturer's instructions. Equal amounts of protein (30 μg/well) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by western blot using specific antibodies against SGLT2 (cat. #37296, Abcam, Bristol, UK) and β-Actin (cat. #47778, Santa Cruz, Dallas, TX, USA). For analysis of the western blot images were analyzed using ImageJ software (NIH Image, Bethesda, MD, USA) for quantifying mesangial expansion and glomerular hypertrophy.

9. Statistical analyses

Data were expressed as the mean ± standard error of mean (SEM). All statistical analyses were conducted using IBM SPSS version 23.0 for Windows (IBM Corp., Armonk, NY, USA). Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA), followed by the Bonferroni's post hoc test; $p < 0.05$ was considered statistically significant.

III. RESULTS

1. Physical and biochemical characteristics of the treatment mice

The body weights of all mice gradually increased during the study period, and the pioglitazone group had significantly greater weight than the other groups beginning at the 4th week of treatment (Figure 1A). The amount of food

consumption was not significantly different between the groups. The combination treatment group experienced the greatest efficacy with 95.3% reduction from baseline ($p < 0.005$ compared to vehicle) (Figure 1B), and showed the most reduced area under the glucose curve (AUC) (Figure 2A, 2B).

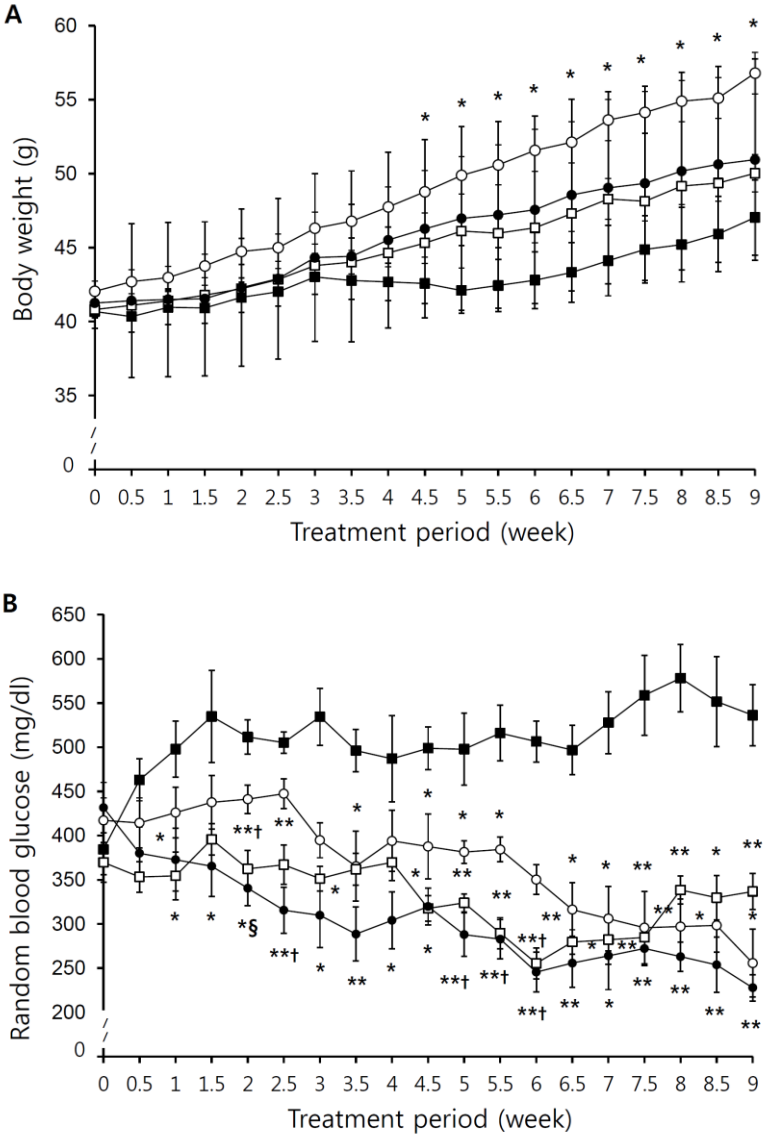


Figure 1. Changes in body weight and blood glucose in vehicle- and drug-

treated *db/db* mice. Graph depicting (A) body weight and (B) random blood glucose concentration in vehicle (black square, ■), pioglitazone (30 mg/kg/day, white circle, ○), dapagliflozin (1 mg/kg/day, white square, □), and combination (30 mg/kg/day of pioglitazone and 1 mg/kg/day of dapagliflozin, black circle, ●) during 9 week of study period. Data are means ± SEM (n = 5-8). *$p < 0.05$ vs vehicle, **$p < 0.001$ vs vehicle, †$p < 0.05$ vs pioglitazone, §$p < 0.05$ vs dapagliflozin by one-way ANOVA and Bonferroni's post hoc test.

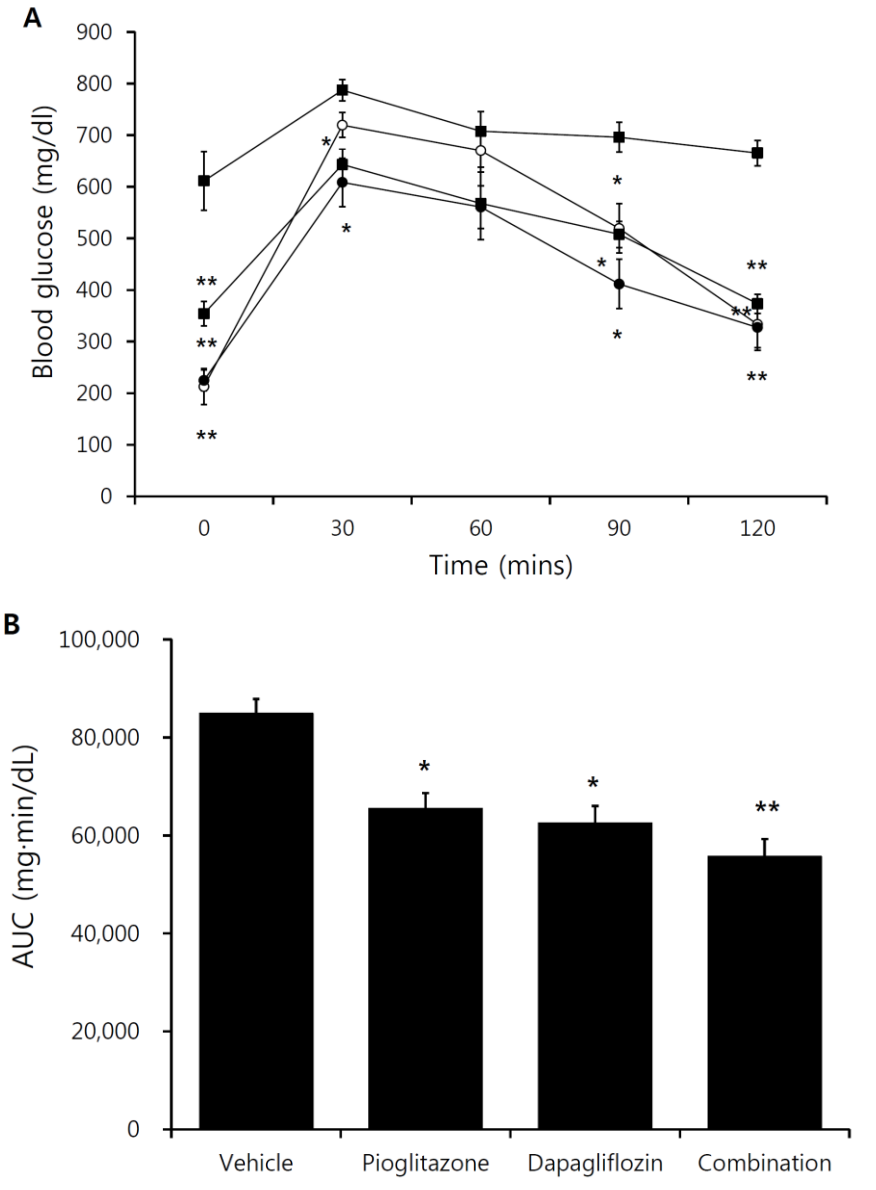


Figure 2. Glucose homeostasis in vehicle- and drug-treated *db/db* mice. (A) Measurement of blood glucose during oral glucose tolerance test of 9 week administration after 6-hr fasting in vehicle (black square, ■), pioglitazone (white circle, ○), dapagliflozin (white square, □), combination (black circle, ●), and (B) area under the curve of the oral glucose concentration. Data are means ±

SEM (n = 5-8). *p < 0.05 vs vehicle, **p < 0.001 vs vehicle by one-way ANOVA and Bonferroni's post hoc test.

Similarly, plasma TG and FFA concentrations were decreased in treatment groups compared to vehicle-treated mice (Figure 3A, 3B).

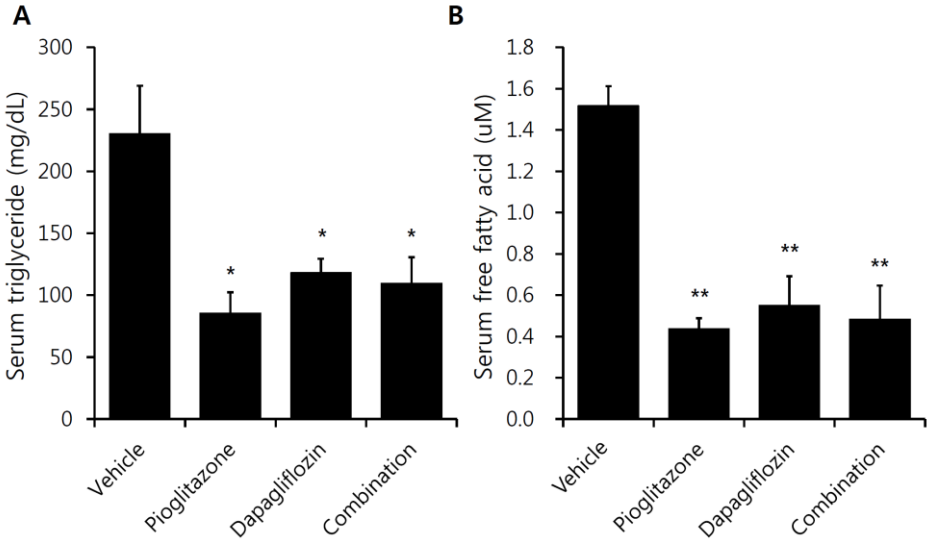


Figure 3. Effects of pioglitazone, dapagliflozin and combination on lipid concentration. (A) Measurement of serum triglyceride and (B) serum free fatty acid after 9 weeks treatment in vehicle (PBS), pioglitazone (30 mg/kg/day), dapagliflozin (1 mg/kg/day), and combination (30 mg/kg/day of pioglitazone and 1 mg/kg/day of dapagliflozin). Data are means ± SEM (n = 5-8). *p < 0.05 vs vehicle, **p < 0.001 vs vehicle by one-way ANOVA and Bonferroni's post hoc test.

2. Renal morphology and albuminuria

Compared to the vehicle-treated group, kidney weights were lowest in the combination group (Figure 4A). Urine albumin to creatinine ratio (UACR) was reduced in all treatment arms, however, no significant difference in UACR was

observed between the treatment groups (Figure 4B).

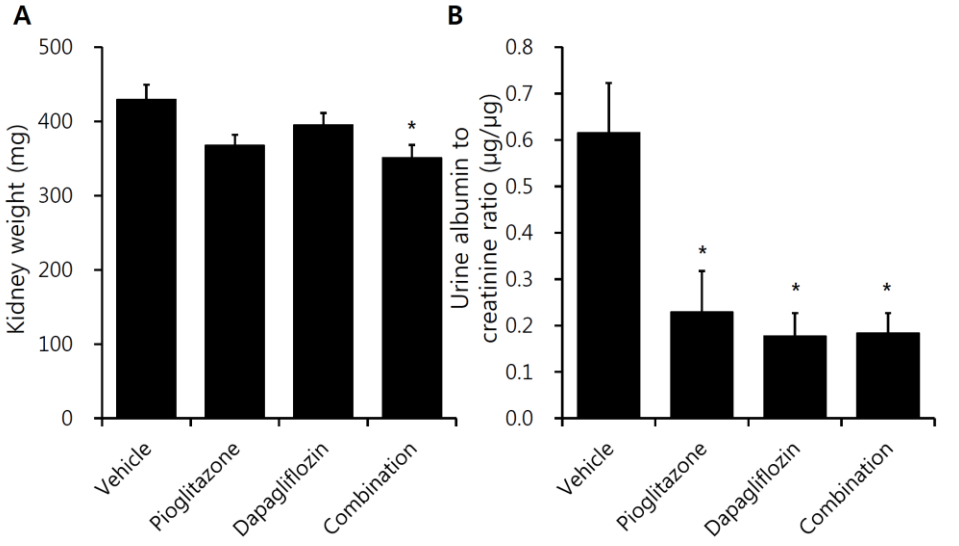


Figure 4. Effects of pioglitazone, dapagliflozin and combination on kidney weight and albuminuria. (A) Measurement of both kidney weight after 9 weeks treatment in vehicle (PBS), pioglitazone (30 mg/kg/day), dapagliflozin (1 mg/kg/day), and combination (30 mg/kg/day of pioglitazone and 1 mg/kg/day of dapagliflozin). (B) Measurements of urine albumin to creatinine ratio after 8 weeks treatment in vehicle (PBS), pioglitazone (30 mg/kg/day), dapagliflozin (1 mg/kg/day), and combination (30 mg/kg/day of pioglitazone and 1 mg/kg/day of dapagliflozin). Data are means ± SEM (n = 5-8). *$p < 0.05$ vs vehicle by one-way ANOVA and Bonferroni's post hoc test.

In immunohistochemistry studies, increased glomerulus size and tubuloglomerularfibrosis were observed in vehicle mice, which were attenuated in the treatment groups (Figure 5A-5D). Pioglitazone and dapagliflozin monotherapy groups showed attenuated glomerular hypertrophy, and both monotherapy groups showed comparable glomerular tuft size (2971.1 ± 62.0 μm^2 for pioglitazone and 2945.2 ± 84.7 μm^2 for dapagliflozin) (Figure 5E). The

combination therapy group showed the greatest reduction in glomerular tuft area (26.4% reduction compared to vehicle, $p < 0.001$). Similarly, the mesangial expansion ratio was lower in the treatment groups, and was lowest in the combination arm (30.0% reduction compared to vehicle, $p < 0.001$) (Figure 5F). Along with these morphologic changes, TUNEL staining demonstrated more apoptosis in the kidney of vehicle mice, which was lower in the pioglitazone and dapagliflozin groups (Figure 5D, 5G). With respect to the three treatment arms, mice treated with the combination showed the lowest number of apoptotic cells in the kidney.

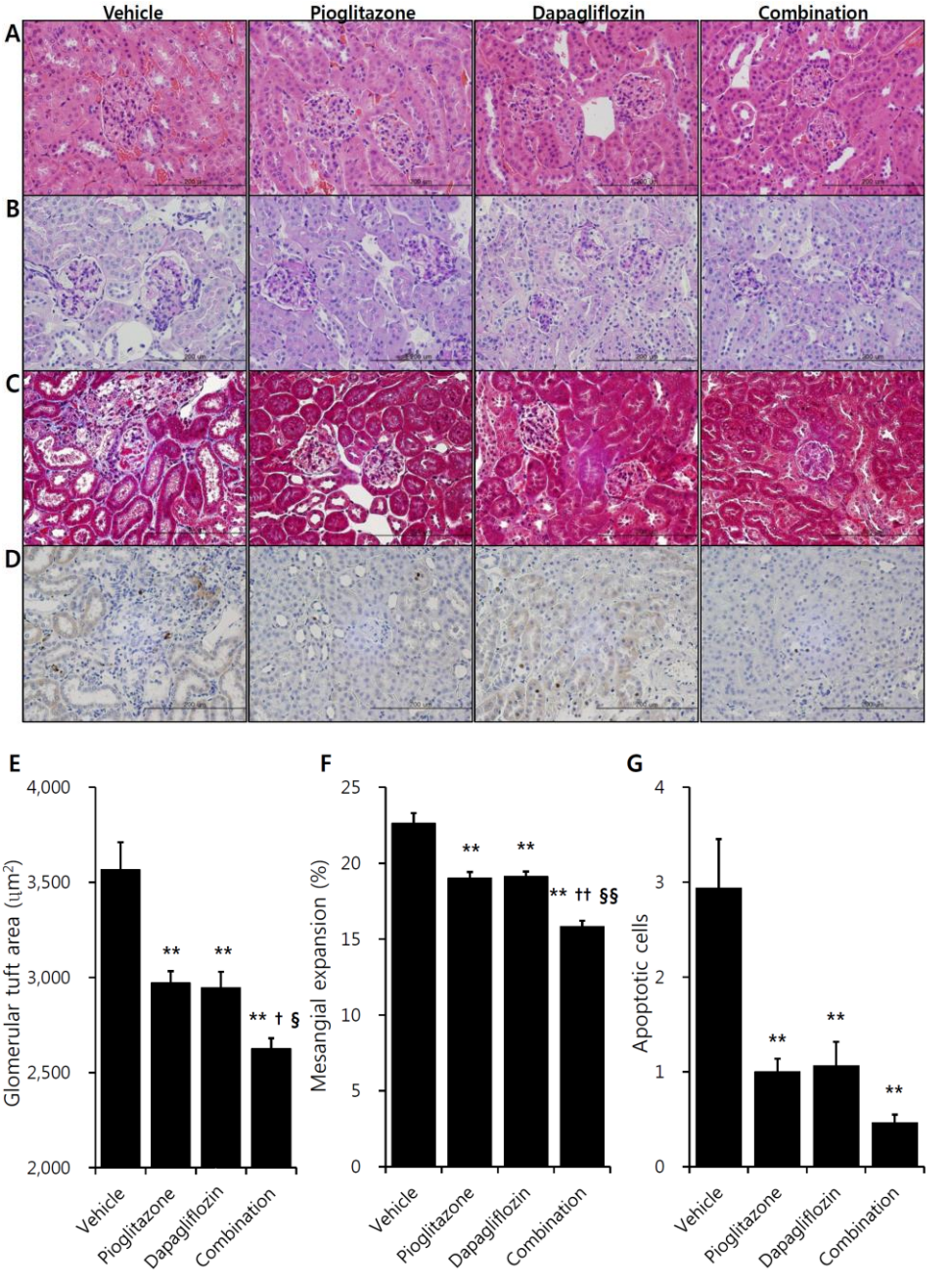
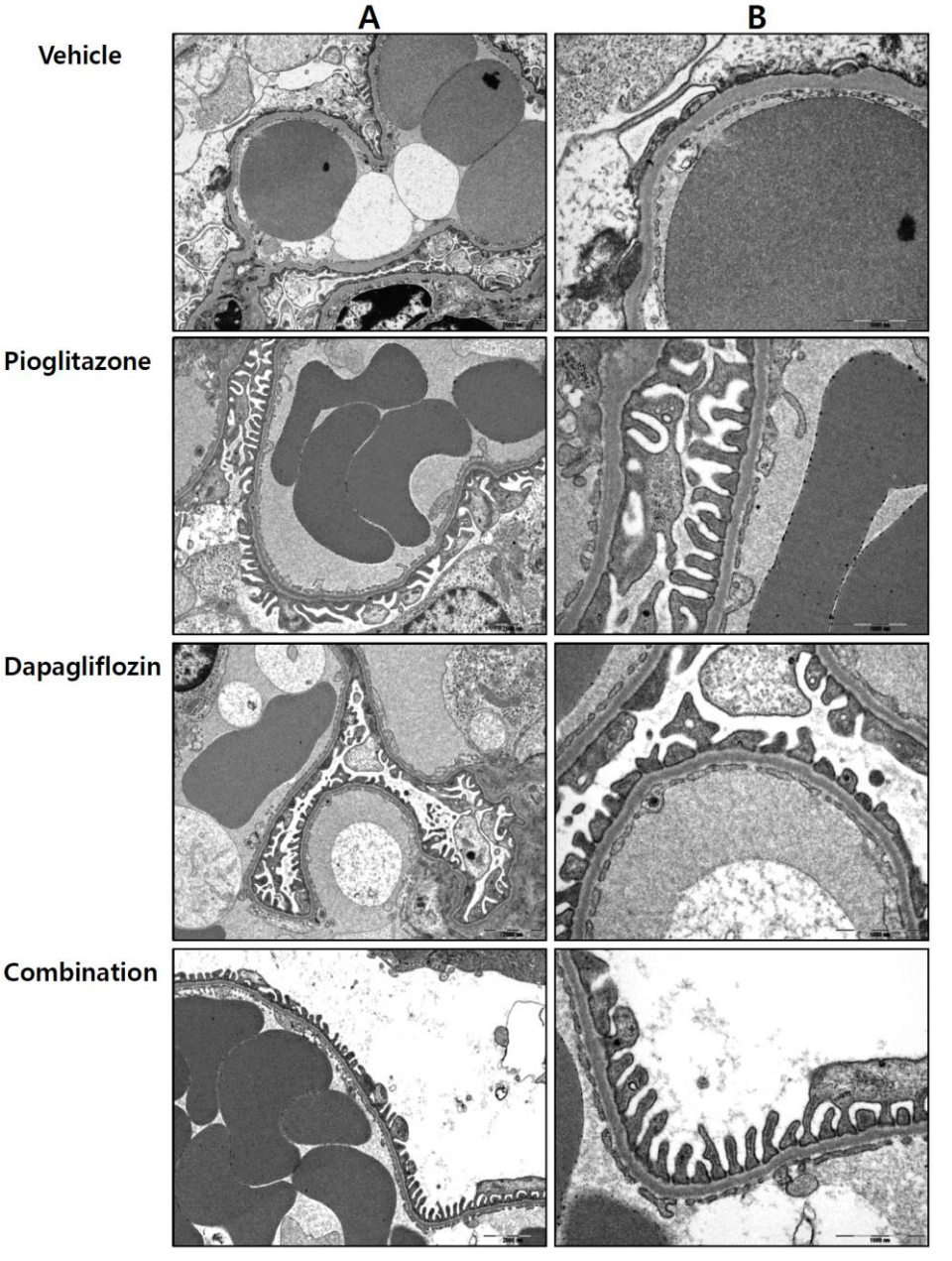


Figure 5. Effects of pioglitazone, dapagliflozin and combination on kidney morphology in immunohistochemistry studies. Renal glomerulus and tubules of 9 weeks treatment in vehicle (PBS), pioglitazone (30 mg/kg/day), dapagliflozin

(1 mg/kg/day), and combination (30 mg/kg/day of pioglitazone and 1 mg/kg/day of dapagliflozin). (A) Hematoxylin and Eosin (H&E) stain, (B) Periodic acid-Schiff (PAS) stain, (C) Masson's trichrome stain, (D) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stain (× 400, bar presents 200 μm). Quantification of (E) glomerular tuft area, and (F) mesangial expansion in PAS stain section minimum of 15 glomeruli per mouse kidney under high power magnification (×400). (G) Counting apoptotic tubular cell in TUNEL stain minimum of 20 randomly selected areas per mouse kidney under high power magnification (×400). Data are means ± SEM (n = 5-8). ** $p < 0.001$ vs vehicle, † $p < 0.05$ vs pioglitazone, †† $p < 0.001$ vs pioglitazone, §§ $p < 0.001$ vs dapagliflozin by one-way ANOVA and Bonferroni's post hoc test.

Electron microscopic examination showed increased irregular thickening of the glomerular basement membrane (GBM) and foot process effacements on glomeruli in the vehicle mice (Figure 6A, 6B). These morphologic changes were attenuated in all treatment groups. Podocyte foot process width was reduced by 45.7% and 44.9%, and GBM thickness was decreased by 37.8% and 37.7% for the pioglitazone and dapagliflozin monotherapy groups, respectively (all $p < 0.001$ compared to vehicle). The combination-treated group showed the best preservation of glomerular morphology with 56.6% reduction in podocyte foot process width and 48.3% reduction in GBM thickness compared to vehicle. In addition, compared to the monotherapy groups, combination treatment resulted in significantly better glomerular structures (GBM thickness $p < 0.05$ compared to pioglitazone, $p < 0.05$ compared to dapagliflozin; foot process width $p < 0.05$ compared to pioglitazone, $p < 0.05$ compared to dapagliflozin) (Figure 6C, 6D).



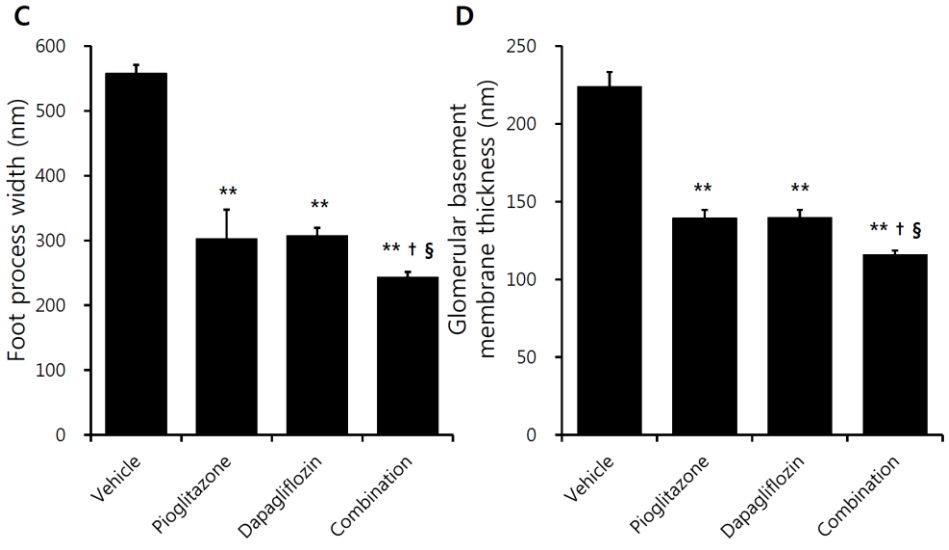


Figure 6. Effects of pioglitazone, dapagliflozin and combination on glomerulus morphology by electron microscope. Glomerulus of 9 weeks treatment in vehicle (PBS), pioglitazone (30 mg/kg/day), dapagliflozin (1 mg/kg/day), and combination (30 mg/kg/day of pioglitazone and 1 mg/kg/day of dapagliflozin). Glomerulus of each treated group (A) ×10,000 magnification, (B) ×30,000 magnification. Quantification of (C) foot process width, and (D) glomerular basement membrane thickness under high power magnification (×30,000). Data are means ± SEM (n = 5-8). ** $p < 0.001$ vs vehicle, † $p < 0.05$ vs pioglitazone, § $p < 0.05$ vs dapagliflozin by one-way ANOVA and Bonferroni's post hoc test.

3. Inflammatory, profibrotic, and renin-angiotensin system-related gene expression

Decreased trends in inflammatory gene expression were observed in the three treatment groups. TGF-β mRNA level was significantly decreased only in the combination-treated mice (Figure 7A). Regarding MCP-1, all treatment groups had decreased expression compared to vehicle; however, there was no significant differences between the groups (Figure 7B). The fibrosis markers, type I and type IV collagen, were significantly decreased in the pioglitazone

monotherapy and combination groups (Figure 7C, 7D) (all $p < 0.05$).

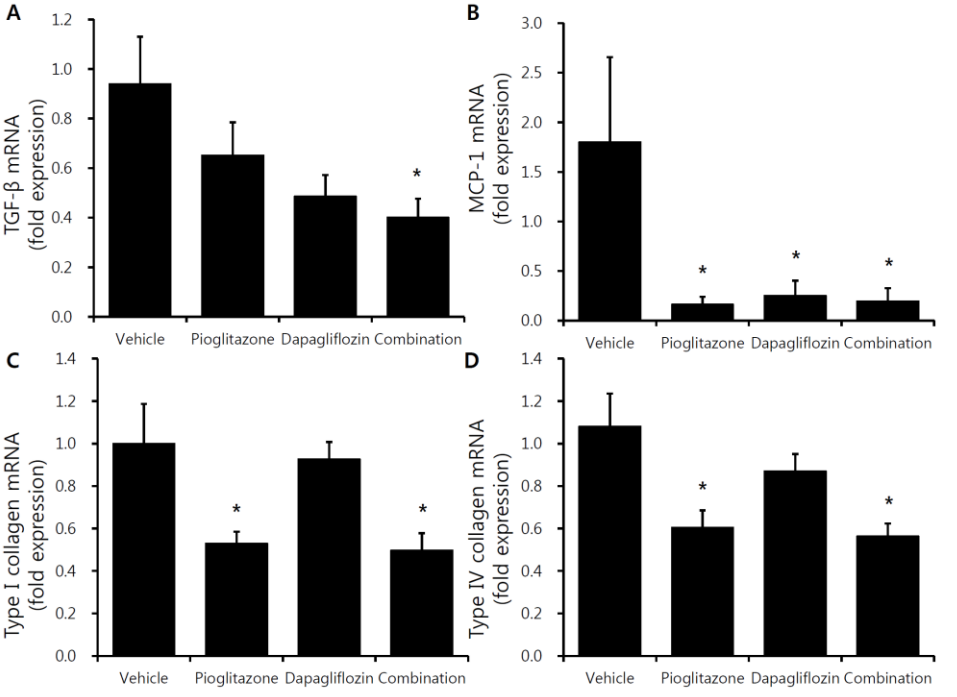


Figure 7. Comparison of inflammatory and profibrotic gene expression in mouse renal cortex. Real-time PCR for 9-week vehicle (PBS)-, pioglitazone (30 mg/kg/day)-, dapagliflozin (1 mg/kg/day)-, and combination (30 mg/kg/day of pioglitazone and 1 mg/kg/day of dapagliflozin)-treated mice' renal cortex. (A) TGF-β (encoding transforming growth factor β), (B) MCP-1 (encoding monocyte chemoattractant protein-1), (C) type 1 collagen, (D) type IV collagen. Data are means ± SEM (n = 5-8). *$p < 0.05$ vs vehicle by one-way ANOVA and Bonferroni's post hoc test.

The renal renin-angiotensin system (RAS) activity tended to decrease in the treatment groups (Figure 8). Dapagliflozin and pioglitazone-treated mice showed lower AGT and renin expression compared to vehicle-treated mice; however, only the combination group had significantly reduced AGT expression

($p < 0.05$).

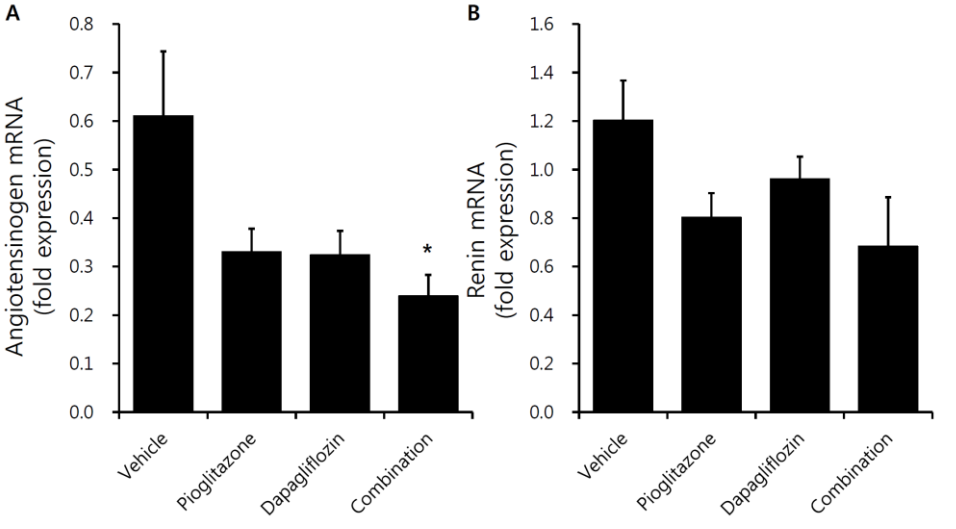


Figure 8. Comparison of angiotensinogen and renin expression in mouse renal cortex. Real-time PCR for 9-week vehicle (PBS)-, pioglitazone (30 mg/kg/day)-, dapagliflozin (1 mg/kg/day)-, and combination (30 mg/kg/day of pioglitazone and 1 mg/kg/day of dapagliflozin)-treated mice' renal cortex. (A) angiogensinogen, (B) renin. Data are means ± SEM (n = 5-8). *$p < 0.05$ vs vehicle by one-way ANOVA and Bonferroni's post hoc test.

4. Effect of treatments on HK-2 cells

As vehicle *db/db* mice showed increased blood glucose and lipid concentrations compared to treated mice, we evaluated high glucose and high lipid-induced inflammation and RAS activation and the direct renoprotective effect of dapagliflozin, pioglitazone, and combination therapy in *in vitro* studies. Compared to cells in normal glucose, the TGF-β/GAPDH mRNA ratios were 6-fold higher in tubular cells exposed to high glucose and palmitate medium ($p < 0.05$) (Figure 9A). This increase in TGF-β mRNA expression was significantly attenuated by single or combination treatment (all $p < 0.05$) and

there was no significance between treatment groups. The levels of MCP-1 and IL-6 protein in conditioned culture medium showed a similar pattern to the TGF-β mRNA expression (Figure 9B, 9C). High glucose and palmitate also significantly induced angiotensinogen and renin mRNA expression in HK-2 cells. The renin expression was lower in all treatment group although the reduction was not significantly different between the treatment groups (Figure 9D). However, the increase in AGT was significantly attenuated (61.0%, $p < 0.05$ vs. vehicle) with combination treatment, which was equivalent to cells in normal glucose (60.6% reduction, $p < 0.05$ vs. vehicle) (Figure 9E). Figure 9F depicts the trend that showed cell survival recovery in the treatment groups; pioglitazone monotherapy and combination treatment had statistically significant recovery ($p < 0.05$ for pioglitazone, $p < 0.05$ for combination). Moreover, the concentration of SGLT2 protein in the HK-2 membrane fraction was not different between the groups ($p > 0.05$) (Figure 10A, B).

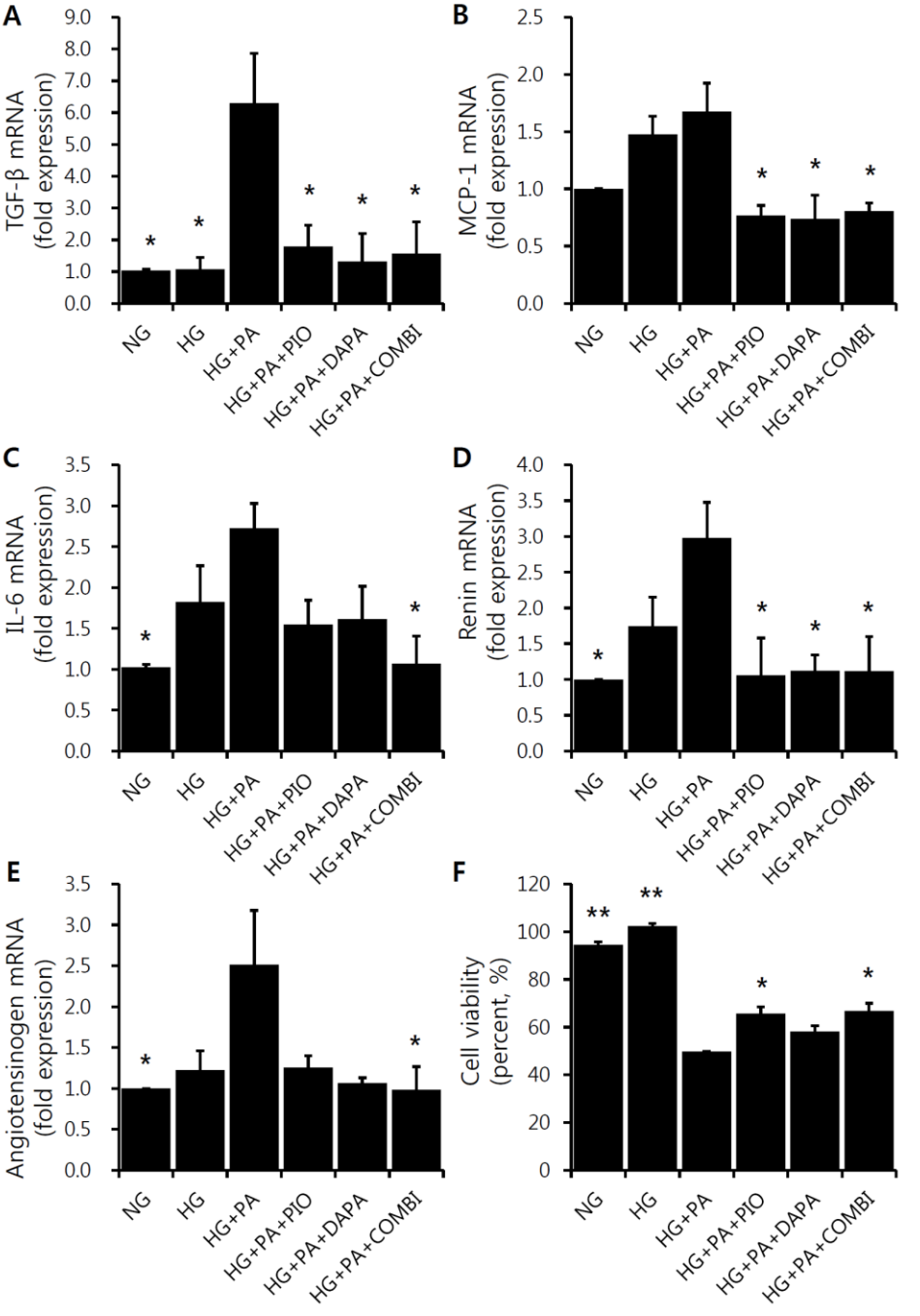


Figure 9. Effect of pioglitazone, dapagliflozin, and combination therapy on inflammatory, profibrotic, and renin-angiotensin system-related gene

exrpression and cell viability of HK-2 cells. HK-2 cell were exposed to either 5.5 mM glucose (normal glucose, NG), 50 mM glucose (high glucose, HG), 0.3 mM palmitic acid (PA), 10 μM pioglitazone (PIO), 10 μM dapagliflozin (DAPA), or 10 μM pioglitazone plus 10 μM dapagliflozin (COMBI). Real-time PCR for 24 hr cultured HK-2 cells for (A) transforming growth factor-β, (B) monocyte chemoattractant protein-1, (C) interleukin-6, (D) angiotensinogen, and (E) renin. (F) MTT assay was performed to determine cell viability in HK-2 cell. Data are means ± SEM ($n \geq 4$). *$p < 0.05$ vs high glucose and palmitic acid group, **$p < 0.001$ vs high glucose and palmitic acid group by one-way ANOVA and Bonferroni's post hoc test.

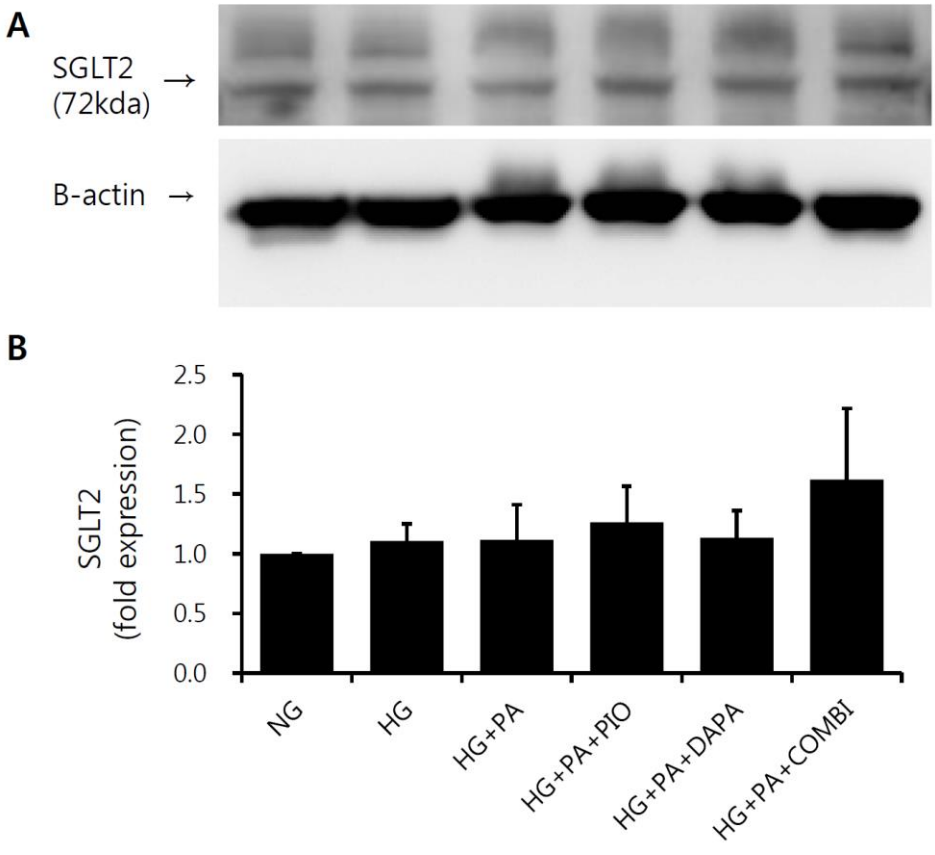


Figure 10. Effect of pioglitazone, dapagliflozin, and combination therapy on

SGLT2 protein expression in human proximal tubular cells (HK-2 cells). HK-2 cell were exposed to either 5.5 mM glucose (normal glucose, NG), 50 mM glucose (high glucose, HG), 0.3 mM palmitic acid (PA), 10 μM pioglitazone (PIO), 10 μM dapagliflozin (DAPA), or 10 μM pioglitazone plus 10 μM dapagliflozin (COMBI). Western immunoblot for 24 hr cultured HK-2 cells for (A) total cell membrane sodium glucose co-transporter 2 (SGLT2) expression and (B) quantitative analysis of SGLT2. Data are means ± SEM (n=4). The concentration of SGLT2 protein in the HK-2 membrane fraction was not different between the groups (p = 0.786).

IV. DISCUSSION

In the present study, we tested the hypothesis that dapagliflozin and pioglitazone combination therapy would prevent T2D-related renal injury and examined its effects on metabolic parameters in a *db/db* mouse model. The results showed that pathological changes in renal cortex, increased albuminuria, and upregulated expression of fibrotic and RAS-related genes in the kidney were ameliorated in the dapagliflozin, pioglitazone, and combination treatment groups. Furthermore, the most attenuation of glomerular hypertrophy, amelioration of fibrosis, and angiotensinogen gene expression was observed in the combination group. We also demonstrated that dapagliflozin, pioglitazone, and combination therapy resulted in reduced inflammatory and RAS-related gene expression in the HK-2 cell experiments. This result suggests that the renoprotective effect of dapagliflozin, pioglitazone, and combination therapy was independent of glucose-lowering efficacy.

Classically, the anti-inflammatory mechanism of TZD on diabetic nephropathy has been well established.[19,20] An *in vitro* study of mesangial cells, pioglitazone attenuated high glucose-induced MCP-1 synthesis, NF-kB activation, and collagen synthesis.[21] In addition, increased antioxidant enzyme induction (Cu-Zn SOD, GSH-Px) in the kidney was ameliorated in a TZD-treated type 1

diabetes animal model, without the glucose-lowering effect, which suggested that TZD has an independent renoprotective effect based on reactive oxygen species inhibition.[22] In our study, upregulated expression of TGF-β, type I and type IV collagens was ameliorated in the pioglitazone monotherapy group. Along with TZD, recent studies support the evidence that SGLT2 inhibitors have a beneficial effect on diabetic nephropathy. SGLT2 in the proximal tubules reabsorb the majority of glucose in the kidney; thus, inhibition of SGLT2 can lower glucose concentration as well as diabetes-related complications. In a 12-week study, dapagliflozin fed to male *db/db* mice resulted in decreased macrophage infiltration by improvement of hyperglycemia in a dose-dependent manner.[23] Similarly, treatment of *db/db* mice with another SGLT2 inhibitor, empagliflozin, resulted in reduced molecular and histological markers of kidney fibrosis and tubule damage (kidney injury molecule-1, neutrophil gelatinase-associated lipocalin) when administrated with metformin.[24] SGLT2 inhibitor treatment in HK-2 cells reversed high glucose-induced inflammatory marker expression (toll-like receptor 4, NF-kB).[25] Furthermore, the EMPA-REG trial outcome provided clinical evidence that SGLT2 inhibitors reduce nephropathy incidence or progression.[26]

However, it seems that dapagliflozin and pioglitazone combination therapy involves other renoprotective mechanisms beyond glucose-lowering effects. Based on our study, although blood glucose levels were lowest in the combination group at the end of treatment, there was no statistically significant difference in blood glucose or oral glucose tolerance test between the three treatment arms. Kidney weights and morphologic changes were mostly improved in the combination group. In addition, only the combination therapy showed significantly reduced TGF-β and AGT mRNA expression. Hyperglycemia and dyslipidemia contribute to renal injury via increased oxidative stress and impaired sodium handling.[27,28] At the early onset of diabetic nephropathy, sodium reabsorption in proximal tubules is upregulated, and

activated tubuloglomerular feedback increases the single nephron glomerular filtration rate, referred as hyperfiltration.[29] As diabetic nephropathy progresses, GBM thickening, mesangial matrix expansion, extracellular matrix accumulation, and tubulointerstitial fibrosis appear. The increase in proximal tubular reabsorption results in tubular hypertrophy and the structural change is mediated by inflammation and growth factors, mainly TGF-β.[30] Along with TGF-β activation, upregulated RAS-related components (renin and angiotensin II) bind to vascular endothelial growth factor and induce renal cell growth and extracellular matrix synthesis.[31] The RAS involvement in stimulating morphogenesis in renal cells is mainly mediated through angiotensin II type 1 receptor.[32] Moreover, angiotensin II mediates transcription of the TGF-β receptor gene, directly upregulating TGF-β or indirectly stimulating MCP-1.[32] The renal RAS pathway also is involved in cardiovascular disease,[33] which is reflected in the cardiovascular benefit of SGLT2 inhibitors.[26]

Although we demonstrated the improvement on renal morphology and renin and angiotensin gene expressions in combination therapy, we could not find any additive effect on blood glucose level. We limited the dapagliflozin dose to 2 mg/day, which was lower than the usual dose of dapagliflozin (10 mg/day) as well as pioglitazone dose (30 mg/day). This might interrupt to determine the glucose lowering effect of combination treatment. Interestingly, the body weights of the combination group were more similar to those of the dapagliflozin group than the pioglitazone group. Considering the small dose of dapagliflozin, the body weight differences between the intervention groups could be meaningful. In addition, our results showed that a small dose of dapagliflozin had equivalent efficacy to pioglitazone on glucose lowering and nephropathy prevention.

The current study provides *in vivo* and *in vitro* evidences that dapagliflozin, pioglitazone, and combination therapy attenuated diabetic nephropathy including albuminuria, renal hypertrophy, and inflammatory and fibrotic

markers. In addition, this investigation demonstrated decreased renal expression of RAS components in treated mice. To our knowledge, the current study is the first to compare the renoprotective effect of SGLT2 inhibitor combination therapy to pioglitazone. Although we did not elucidate the synergistic mechanism of combination therapy, we observed a tendency toward lower profibrotic and inflammatory gene expression in the combination therapy group, which might have been due to the additive effect of those two medications. With respect to body weight, the combination therapy would be expected to provide complementary effects. Moreover, our *in vitro* study results suggest dapagliflozin and pioglitazone combination therapy can ameliorate diabetic nephropathy independently of reducing blood glucose, rather than as a secondary effect to reducing hyperglycemia.

V. CONCLUSION

The current study showed that dapagliflozin, pioglitazone, and combination therapy significantly attenuated diabetic nephropathy progression, and that the renoprotective effect was magnified by combination treatment. Therefore, dapagliflozin and pioglitazone combination therapy could be an effective option to prevent diabetic nephropathy.

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ABSTRACT (IN KOREAN)

다파글리플로진과 피오글리타존 병합요법이 당뇨병성 신증에 미치는 효과

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한유진

다파글리플로진과 피오글리타존의 혈당강하 및 항염증효과는 알려져 있지만 이 두 가지 약제의 병합요법이 당뇨병성 신증에 미치는 영향에 대해서는 현재까지 연구된 바가 없다. 9주의 *db/db* 마우스를 다음과 같이 4개의 군으로 무작위 배정하여 (1) 위약, (2) 다파글리플로진, (3) 피오글리타존, 또는 (4) 다파글리플로진과 피오글리타존 병합제를 9주간 위관영양으로 주입하였다. 세포 실험으로서 근위관 상피세포를 5.5 ㎜ 포도당 혹은 50 ㎜ 포도당과 팔미트산이 들어있는 배지에 배양하고 다파글리플로진, 피오글리타존 단독 또는 병합요법을 시행 후 염증 관계 단백질, 레닌안지오텐신 관련 인자의 발현과 세포 생존율을 확인하였다. 병합 요법군에서 신장의 사구체 면적과 사구체간질의 팽창이 가장 적었으며 신장 무게도 가장 가벼웠다. 사구체의 족세포 발돌기의 너비와 사구체 기저막의 두께를 비교해보았을 때, 위약군에 비해 치료군에서 그 크기가 줄어들어 있었으며, 병합군에서 가장 많이 감소되어 있었다 (p

< 0.05). 또한 병합군에서 MCP-1과 I형, IV형 콜라겐 단백질과 안지오텐시노겐의 발현이 가장 감소되었으며, 세포실험에서도 병합처리를 한 군에서만 안지오텐시노겐과 IL-6의 발현이 가장 감소되었다 (p < 0.05). 결과적으로 다파글리플로진과 피오글리타존 사용은 *db/db* 마우스에서 신기능 유지에 도움을 주며 병합요법은 가장 큰 효과를 나타내었다. 이러한 결과는 제2형 당뇨병 환자에서 다파글리플로진과 피오글리타존의 병합요법이 당뇨병성 신증의 진행을 예방할 수 있을 것이라는 것을 시사한다.

핵심되는 말: 다파글리플로진, 피오글리타존, 당뇨병성신증, 2형 당뇨병