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Effect of dual inhibition of DPP4 and SGLT2 on tacrolimus-induced diabetes mellitus and nephrotoxicity in a rat model

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Sodium/glucose co-transporter-2 inhibitor (SGLT2i) or dipeptidyl peptidase IV inhibitor (DPP4i) is a newer anti-diabetic drug in type II diabetes mellitus (DM), but their use in tacrolimus (TAC)-induced DM is still undetermined. We performed this study to evaluate the effect of these two drugs in TAC-induced DM and nephrotoxicity in ex vivo and in vivo. In the experimental Sprague Dawley rat model of TAC-induced DM and nephrotoxicity, dual inhibition of DPP4 and SGLT2 significantly decreased blood glucose level, HbA1C and increased plasma insulin levels and pancreatic islet size compared with each drug. In the kidney, dual inhibition improved renal function decreased interstitial fibrosis and profibrotic cytokines compared with DPP4i and SGLT2i alone. Increased oxidative stress by TAC was remarkably decreased with DPP4i or SGLT2i in serum, pancreatic and renal tissues and this decrease was much more significant in the combination group. In in vitro study, TAC decreased the cell viability of human kidney-2(HK-2) cells and insulin-secreting beta-cell-derived line(INS-1) cells. SGLT2i protected TAC-induced cell death in HK-2 cells, but not in INS-1 cells. The addition of DPP4i to SGLT2i compensated for a lack of protective effect of SGLT2i on INS-1 cells. This finding provides the rationale for the combined treatment of SGLG2i and DPP4i in TAC-induced DM and nephrotoxicity.

KEYWORDS
DPP4 inhibitor, PTDM, SGLT2 inhibitor, tacrolimus

1 | INTRODUCTION

New-onset diabetes after transplant (PTDM) is a significantly important metabolic complication and is closely associated with poor patient and graft survival.[1,2] Tacrolimus (TAC) is the most popular immunosuppressant drug, but long-term administration of TAC is associated with a high risk of developing PTDM.[3,4] The mechanisms underlying the development of TAC-induced DM are poorly characterized, but a direct injury to pancreatic islet cells and impaired insulin signaling by TAC are regarded as the main features of its pathogenesis; oxidative stress also plays a significant role in the process of TAC-induced pancreatic islet dysfunction.[5]

Abbreviations: 8'-OHdG, 8-hydroxy-2'-deoxyguanosine; AKI, acute kidney injury; AUCg, area under the curve of glucose; Clcr, creatinine clearance; DM, diabetes mellitus; DPP4, dipeptidyl peptidase 4; DPP4i, dipeptidyl peptidase 4 inhibitor; EM, empagliflozin; EXD, exendin-4; GG, gemigliptin; H2-DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; HbA1C, hemoglobin A1C; HK-2 cell, human kidney-2 cell; INS-1 cell, insulin-secreting beta-cell; IPGTT, intraperitoneal glucose tolerance test; PI, propidium iodide; PTDM, new onset diabetes after transplantation; ROS, reactive oxygen species; s.c., subcutaneously; Scr, serum creatinine; SD, standard deviation; SE, standard error; SGLT2, sodium/glucose cotransporter-2; SGLT2, sodium/glucose cotransporter-2 inhibitor; TAC, tacrolimus; VH, vehicle.

The guidelines for PTDM management are based on type 2 DM.[6] Among these, dipeptidylpeptidase-4 inhibitors (DPP4i) and sodium-glucose cotransporter-2 inhibitors (SGLT2i) have been adopted in clinical practice. In addition to their effective control of hyperglycemia, a recent clinical trial revealed that SGLT2i and DPP4i have cardiovascular and renal protective effects in type 2 DM.[7-9] Therefore, dual inhibition of DPP4 and SGLT may be useful in renal transplant patients with PTDM. It is, therefore, anticipated that dual inhibition of SGLT2 and DPP4 may have advantages not only in glycemic control but also in renoprotection.[10] Therefore, we aimed to assess the effect of combined SGLT2i and DPP4i treatment on hyperglycemia and TAC-induced organ injury in vivo and in vitro.

2 | MATERIALS AND METHODS

2.1 | Animals and drugs

The Animal Care and Use Committee of the Catholic University of Korea approved the experimental protocol (CUMC-2017-0181-03), and all procedures performed in this study were in accordance with ethical guidelines for animal studies. Eight-week-old male Sprague Dawley rats (Charles River Technology, Seoul, Korea), initially weighed at 220–230 g, were housed in cages (Nalge Co., Rochester, NY) in a temperature- and light-controlled environment at the Animal Care Facility of the Catholic University of Korea. The rats received a low-salt diet (0.05% sodium, Teklad Premier, Madison, WI). Tacrolimus (TAC, Prograft, Astellas Pharma Inc., Ibaraki, Japan) was diluted in olive oil (Sigma, St. Louis, MO) to a final concentration of 1 mg/ml. Empagliflozin (EM), an SGLT2i, was provided by Boehringer Ingelheim Pharma GmbH & Co. KG (Ingelheim, Germany) and was diluted in drinking water to a final concentration of 10 mg/kg.[4] Gemigliptin (GM), a DPP4i, was kindly supplied by LG Life Sciences (Seoul, Korea) and was diluted in drinking water to a final concentration of 20 mg/kg/day.

2.2 | Experimental design

After acclimatization and administration of a low-salt diet for 1 week, weight-matched rats were randomized to 5 groups containing 12 rats each and were treated daily with TAC (1.5 mg/kg, subcutaneously [s.c.]) or vehicle (VH, olive oil, 0.3 mL, s.c.) for 3 weeks. After confirming the development of TAC-induced DM, EM (10 mg/kg by oral gavage), GG (20 mg/kg by oral gavage), and a combination of EM and GG (EM 10 mg/kg and GG 20 mg/kg by oral gavage) were administered for 3 weeks. The dose and duration of treatment were chosen based on previous reports.[11,12]

2.3 | Animal maintenance and monitoring

Rats were pair-fed and their body weight was monitored daily. After the treatment period, animals were housed individually in metabolic cages (Tecniplast, Gazzada, Italy), and their urine volume and water intake were measured over 24 h. Animals were anesthetized, and blood samples and tissue specimens were obtained. Analyses of serum electrolyte concentrations and urine glucose levels were performed using enzymatic colorimetric methods (Modular DPP system; Roche, Hamburg, Germany). Serum creatinine (Scr) level was measured using a quantitative enzyme colorimetric method (Stanbio Laboratory, Boerne, TX). Creatinine clearance (Clcr) was calculated from 24 h of urine collection and serum using a standard formula. The TAC level of whole blood was measured using liquid chromatography-mass spectrometry/mass spectrometry.[13]

2.4 | Preservation of kidney and pancreatic tissues and isolated islets

Kidney and pancreatic tissue were preserved by in vivo perfusion through the abdominal aorta. The animals were perfused with 0.01 mol/L phosphate-buffered saline, and the dissected kidney and pancreases were immersed in periodate-lysine-2% paraformaldehyde solution and embedded in paraffin.

2.5 | The intraperitoneal glucose tolerance test and plasma insulin level

The intraperitoneal glucose tolerance test (IPGTT) was performed at the end of the treatment period, as previously described,[14] and the area under the curve of glucose (AUCg) was calculated by trapezoidal estimation from the values obtained in the IPGTT. The plasma insulin level was measured in duplicate using an enzyme-linked immunosorbent assay kit (Millipore Corporation, St. Charles, MO).

2.6 | Measurement of pancreatic beta cell area

A minimum of 20 files per section were assessed using a color image analyzer (TDI Scope EyeTM Version 3.0; Olympus, Japan). Briefly, captured images from immunohistochemistry with insulin were quantified using the Polygon program by measuring the pancreas area seen to contain insulin-positive area except for vacuoles when viewed under ×200 magnifications. Histopathologic analysis was performed on randomly selected fields of the pancreas section by a pathologist blinded to the identity of the treatment groups. Insulin-positive cells were evaluated by counting approximately 20 randomly selected non-overlapping islets for the animals in each group.

2.7 | Measurement of interstitial fibrosis and glomerular injury in the kidney

Histological assessment of tubule interstitial fibrosis (TIF) in trichrome-stained tissue sections was performed as described

previously.[15] The extent of fibrosis was estimated by counting the proportion of injured area per field using a polygon program.

2.8 | Immunohistochemistry

Immunohistochemistry was performed as previously described.[16] Primary antibodies against insulin (Zymed, CA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were used. Twenty different fields in each section at 9400 magnification were analyzed using a color image analyzer (TDI Scope Eye Version 3.0; Olympus, Japan).

2.9 | Immunoblot analysis

Immunoblot analysis was performed as previously described.[17] From tissue lysates of the renal cortex, E-cadherin, α-SMA, β-igh3 (BD Biosciences, CA), and β-actin (Sigma) were detected by incubating samples for 12 h with specific antibodies. An image analyzer (Quantity One version 4.4.0; Bio-Rad, CA) was used to analyze the immunoblot.

2.10 | Measurement of serum 8-hydroxy-2'-deoxyguanosine

Oxidative DNA damage was evaluated based on the level of DNA adduct 8-OHdG in serum samples using a competitive enzyme-linked immunosorbent assay (Cell Biolabs, CA).

2.11 | Measurement of cell viability

We evaluated the direct effect of additive or independent treatment with EM, EXD on TAC-induced injury using human kidney-2 (HK-2) proximal tubule cells and insulin-secreting beta-cell-derived line (INS-1) cells. Cells were treated with different concentrations (1 ng/ml–1000 mg/ml) of EM and 10 ng/ml EXD for 12 h during TAC-induced toxicity. HK-2 cells and INS-1 cells were seeded into 96-well plates at a density of 2.5×10^4 and 5×10^4 cells/well, respectively, and pre-incubated for 24 h in an incubator at 37°C. After 24 h, the culture medium was changed to serum-free medium containing TAC (60 ug/mL) and/or Em (1, 10, 100, and 1000 nM), Exd (10 nM). Cell viability was assayed using a cell counting kit-8 assay kit (Dojin Laboratories, Japan) according to the manufacturer's protocol.[18] Cell viability measured using a cell counting kit-8 (CCK-8, CK04; Dojindo Molecular Technologies, MD) assay kit. One day after seeding, HK-2 and INS-1 cells were subjected to various treatments for the specified periods. Prior to the completion of these treatments, CCK-8 or propidium iodide (PI; 556463; BD Biosciences, CA) solution was added to each well or trypsinized cells according to the manufacturer's protocol. Following the addition of CCK-8, absorbance was measured at 450 nm using a Versa Max ELISA Reader (Molecular Devices, CA). The PI-stained dead cells were detected using a FACS Calibur flow cytometer (BD Biosciences). Experiments were performed in triplicates, 3 times in each experiment.

2.12 | Flow cytometry

Flow cytometry was performed to assess the production of reactive oxygen species (ROS). HK-2 and INS-1 cells were seeded into 6-well plates at a density of 2.5×10^5 cells/well and pre-incubated for 24 h at 37°C in an incubator. After 24 h, the culture medium was changed to serum-free medium containing TAC (50 lg/ml) and Em (1, 10, 100, and 1000 nM), Exd (10 nM). The method was performed as previously described.[19]

2.13 | Statistical analysis

The data are expressed as means ± standard error of at least three independent experiments. Multiple comparisons between groups were performed by one-way ANOVA with the Bonferroni post hoc test (SPSS software version 19.0; IBM, NY). Statistical significance was set at $p < .05$.

3 | RESULTS

3.1 | Effect of combined treatment with SGLT2 inhibitor and DPP4 inhibitor on basic parameters

Table 1 lists the changes in the functional basic parameters. Following 3 weeks of TAC treatment, EM treatment significantly decreased body weight independently and in combination with GG when compared with the VH and TAC group, whereas there was no significant change following treatment with GG alone (Table 1). Urine volume (UV) was increased after 3 weeks of treatment of TAC, but the addition of EM increased UV more prominently in EM and combined treatment EM and GG, whereas GG treatment showed no prominent change compared to the TAC group. There was no significant difference in the electrolyte content and trough level of TAC in the whole blood.

3.2 | Effect of combined treatment with SGLT 2 inhibitor and DPP4 inhibitor on hyperglycemia and plasma insulin levels in TAC-induced DM

TAC treatment significantly increased the AUCg compared with the VH (314 ± 20 vs. 154 ± 5 mg/dl/min, $p < .05$), whereas EM or GG treatment attenuated the increase in AUCg induced by TAC. More

TABLE 1 Basic parameters in each group

	Vh	Tac	Tac + EM	Tac + GG	Tac + EM + GG
△BW (g)	95 ± 10	55 ± 8[a]	48 ± 8[a]	72 ± 5	61 ± 8
UV (ml/day)	11 ± 2	32 ± 6[a]	49 ± 4[a,b]	30 ± 4[a,c]	49 ± 7[a,b,d]
Na^+ (mmol/L)	143 ± 0.8	139 ± 0.8	142 ± 0.8	141 ± 1.3	141 ± 1.2
K^+ (mmol/L)	4.0 ± 0.2	4.0 ± 0.1	3.7 ± 0.1	4.0 ± 0.3	4.5 ± 0.4
Cl^- (mmol/L)	94 ± 1.0	95 ± 1.0	96 ± 0.7	98 ± 1.5	98 ± 1.5
Tac con (ng/ml)	—	10.5 ± 1.7	9.8 ± 1.7	10.3 ± 1.5	11.0 ± 2.3

Note: Values are means ± standard error.

Abbreviations: △BW, changes of body weight; Em, empagliflozin; GG, gemigliptin; TAC, tacrolimus; TAC con, TAC concentration; UV, urine volume.

[a] $p < .05$ vs. Vh.

[b] $p < .05$ vs. Tac.

[c] $p < .05$ vs. Tac + EM.

[d] $p < .05$ vs. Tac + GG.

importantly, the addition of EM to GG was more effective in attenuating the AUCs compared with EM or GG alone (TAC + EM; 249 ± 17, TAC + GG; 244 ± 23 vs. TAC + EM + GG; 223 ± 6 mg/dl/min, $p < .05$) (Figure 1A,B). TAC significantly decreased the plasma insulin level (0.40 ± 0.05 vs. 1.39 ± 0.10 ng/ml, $p < .05$), but addition of EM to GG recovered the plasma insulin level (TAC + EM; 0.76 ± 0.16, TAC + GG; 0.92 ± 0.10 vs. TAC + EM + GG; 1.26 ± 0.16 ng/ml, $p < .05$) (Figure 1D).

3.3 | Effect of combined treatment with SGLT inhibitor and DPP4 inhibitor on pancreatic islet size

The pancreatic beta-cell area was evaluated using immunohistochemistry. The TAC group had smaller islets and a lower intensity of insulin staining within islets compared with the VH group (VH 32.4 ± 2.6 μm^2 vs. TAC 11.1 ± 8.8 μm^2, $p < .05$, Figure 2A and B). Although the shrinkage of islets induced by TAC was not prevented by EM or GG treatment alone (TAC + EM; 15.2 ± 2.0, TAC + GG; 15.5 ± 1.7 μm^2), the addition of EM to GG rescued the shrinkage of islets (22.4 ± 2.1 μm^2, $p < .05$).

3.4 | Effect of combined treatment with EM and GG on renal function and urinary glucose excretion

Figure 3 shows the effect of EM, GG, and combined treatment with EM and GG on TAC-induced renal dysfunction. The TAC group demonstrated increased Scr (0.6 ± 0.1 vs. 0.3 ± 0.1 mg/dl, $p < .05$) and decreased Clcr compared to the VH group (0.4 ± 0.1 vs. 1.4 ± 0.1 ml/min/100 g, $p < .05$). However, the addition of or independent treatment with EM or GG suppressed the elevation of serum creatinine levels (TAC + EM 0.4 ± 0.1, TAC + GG 0.4 ± 0.1 mg/dl); the combined use of EM and GG significantly reversed the changes (0.3 ± 0.1 mg/dl, $p < .05$) (Figure 3A, B). Additionally, urinary glucose excretion was significantly higher in the EM treatment group, alone and in combination with GG (TAC 196 ± 101 vs. TAC + EM 1446 ± 300, TAC + EM + GG 1518 ± 345, both $p < .05$).

3.5 | Effect of combined treatment with SGLT and DPP4 inhibitors on renal interstitial fibrosis

TAC was associated with an increased TIF score (12.1 ± 1.7 vs. 0 ± 0%, $p < .05$) compared to the VH group (Figure 4A, B). The addition of or independent treatment with EM or GG reduced the TIF score (EM; 4.9 ± 1.2, GG: 4.5 ± 0.6, $p < .05$). Furthermore, combination EM to GG more prominently decreased the TIF score (1.9 ± 0.4, $p < .05$).

We further evaluated the effect of combined treatment on cytokines involved in interstitial fibrosis. In contrast, the addition of or independent treatment with EM or GG increased E-cadherin expression compared to that in the TAC group (Figure 5A,B, $p < .05$). Meanwhile, TAC significantly increased α-SAM (TAC 2.0 ± 0.0 vs. VH 1.0 ± 0.1, $p < .05$) and β-igh3 expression (TAC 1.8 ± 0.0 vs. VH 1.0 ± 0.1, $p < .05$) (Figure 5C). The addition of, or independent treatment with, EM or GG decreased α-SAM and β-igh3 expression; these changes were prominent in the combined treatment group (EM + GG) ($p < .05$) (Figure 5D).

3.6 | Effect of combined treatment with SGLT inhibitor and DPP4 inhibitor on TAC-induced oxidative stress

Accumulated evidence indicates that TAC treatment increases oxidative stress in vivo and in vitro.[4,11] The TAC-induced increase in serum levels of 8-OHdG was lowered by the addition of or independent treatment with EM or GG. Interestingly, only the addition of EM to GG significantly suppressed the elevation of serum 8-OHdG level (Figure 6, $p < .05$).

In this study, immunohistochemistry was performed to evaluate oxidative stress by detecting the levels of 8-OHdG. TAC treatment significantly increased the production of 8-OHdG in

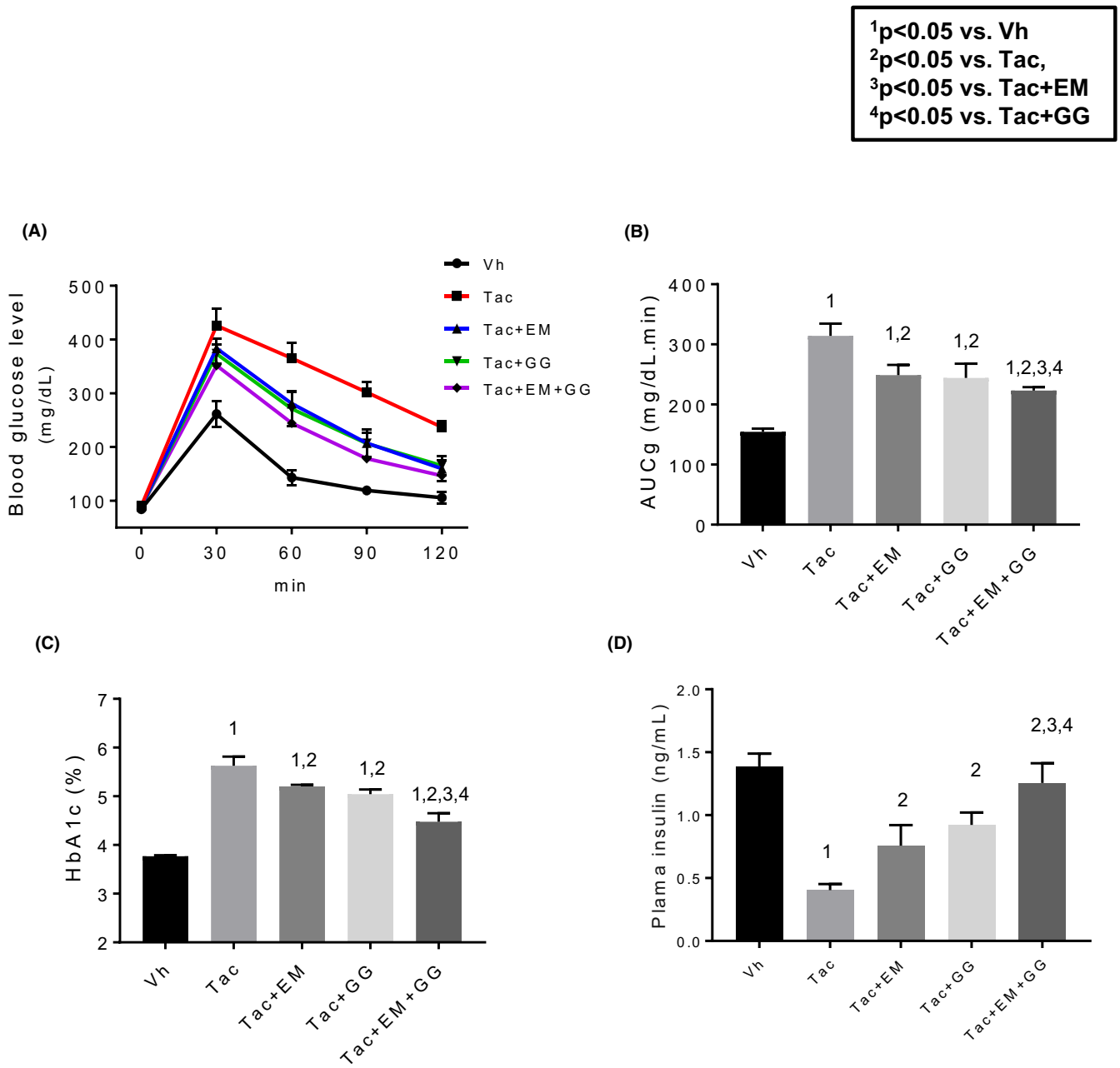

FIGURE 1 Effects of combined treatment of SGLT2 inhibitor and DPP4 inhibitor Tac-induced DM. (A) Intraperitoneal glucose tolerance test (IPGTT). (B) Area under the curve of glucose (AUCg). (C) Hemoglobin A1C (HbA1C). (D) Plasma insulin concentrations. Results are expressed as mean ± SE and are representative of four independent experiments. [1]$p < .05$ vs. Vh, [2]$p < .05$ vs. Tac, [3]$p < .05$ vs. Tac + EM, [4]$p < .05$ vs. Tac + GG

both pancreatic islet (10.5 ± 1.6 vs. 0.6 ± 0.3 $mm^2 \times 10^4$, $p < .05$) (Figure 7A,B) and kidney tissue (55.4 ± 3.4 vs. 1 ± $mm^2 \times 10^4$, $p < .05$) (Figure 8A,B), and this effect could be suppressed by the addition of either EM or GG (pancreatic islet—TAC + EM 7.6 ± 0.5, TAC + GG 6.2 ± 1.0 $mm^2 \times 10^4$, both $p < .05$; kidney—TAC + EM 41.7 ± 1.8, TAC + GG 40.8 ± 2.7 $mm^2 \times 10^4$, both $p < .05$) (Figure 8 A, B). Moreover, the addition of EM to GG was more effective in reducing the levels of serum 8-OHdG than was each drug alone (pancreatic islet; TAC+EM+GG 3.5 ±1.2 $mm^2 \times 10^4$, $p < .05$; kidney; 29.6 ± 4.0 $mm^2 \times 10^4$, $p < .05$).

3.7 | Effect of combined treatment with an SGLT2 inhibitor and exendin-4 on a pancreatic islet and renal cell viability

We evaluated the direct effect of additive or independent treatment with EM or EXD on TAC-induced injury using HK-2 proximal tubule cells and INS-1 cells. HK-2 cell viability decreased with TAC, whereas EM improved cell viability regardless of the concentration. Administration of Exd improved cell viability more than EM, and the combination of EM and Exd resulted in maximum improvement in

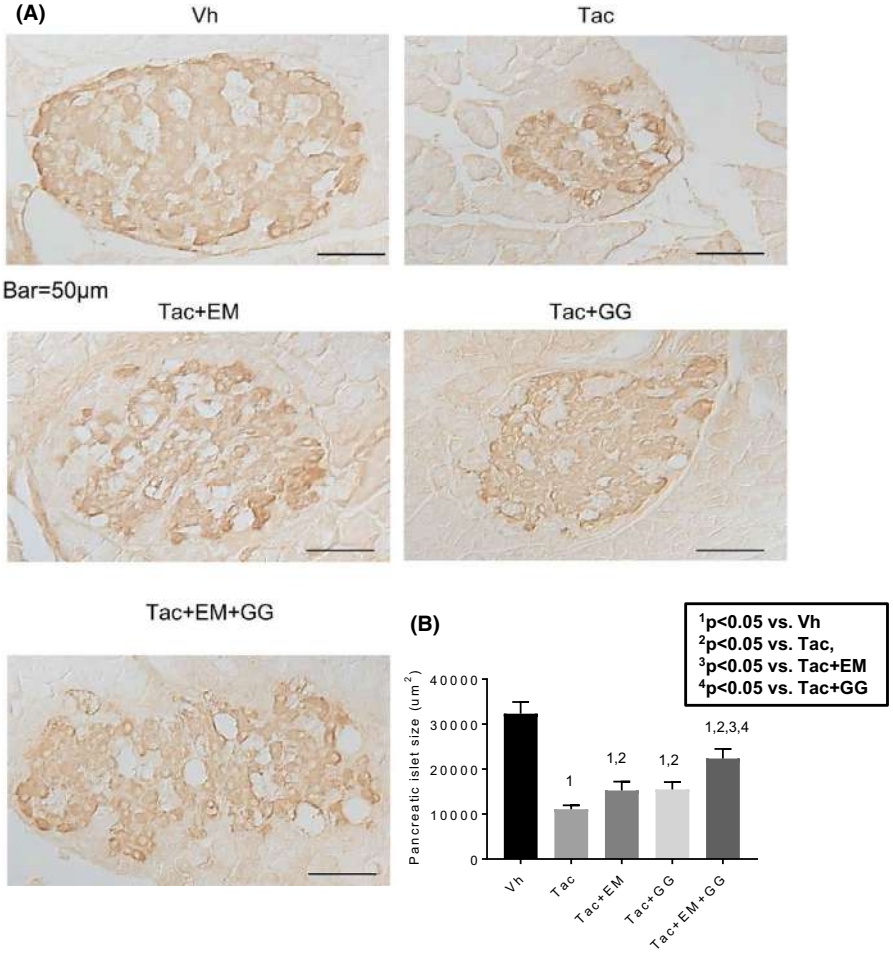

FIGURE 2 Effect of combined treatment of SGLT2 inhibitor and DPP4 inhibitor on pancreatic islet size in Tac-induced DM. (A) Immunohistochemistry of insulin in the pancreas. Original magnification, ×400. (B) Pancreatic islet size ($\mu m^2 \times 10^3$). Scale bars represent 50 μm. [1]$p < .05$ vs. Vh, [2]$p < .05$ vs. Tac, [3]$p < .05$ vs. Tac + EM, [4]$p < .05$ vs. Tac + GG

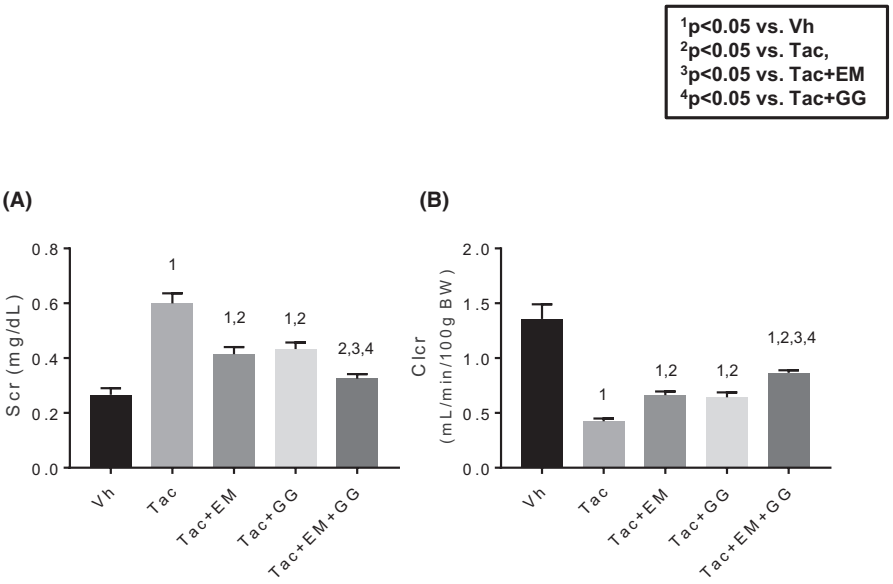

FIGURE 3 Effect of combined treatment of SGLT2 inhibitor and DPP4 inhibitor on Tac-induced renal dysfunction. ClCr, creatinine clearance; Scr, serum creatinine. [1]$p < .05$ vs. Vh, [2]$p < .05$ vs. Tac, [3]$p < .05$ vs. Tac + EM, [4]$p < .05$ vs. Tac + GG

cell viability (Figure 9A). In contrast, INS-1 cell viability was reduced in the TAC group, but the addition of EM did not rescue this change. Nevertheless, Exd treatment recovered the cell viability, and a combination of EM and Exd resulted in maximum improvement in INS-1 cell viability. The addition of EM increased the viability of HK-2 cells but not INS-cells (Figure 9B).

3.8 | Effect of combined treatment with EM and Exd on ROS production and cell death in HK-2 cells

We further evaluated the direct effect of EM or Exd on HK-2 cells TAC-induced oxidative stress and cell death. Figure 10A and B show that EM or Exd decreased the intracellular ROS production,

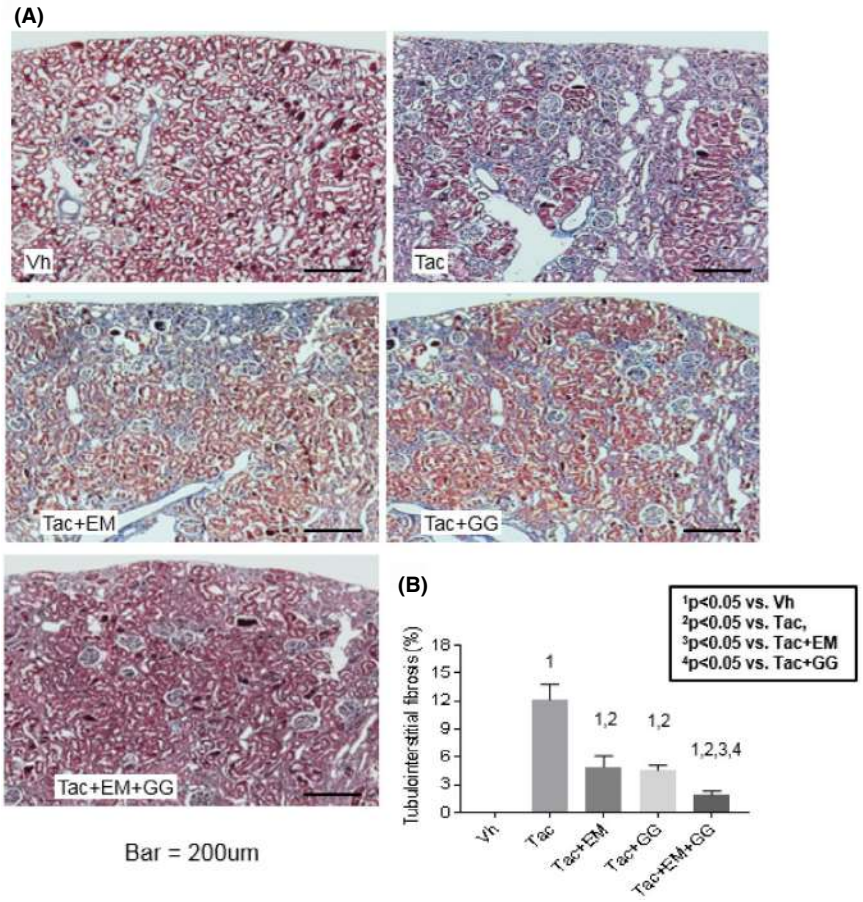

FIGURE 4 Effect of combined treatment of SGLT2 inhibitor and DPP4 inhibitor on Tac-induced interstitial fibrosis in kidney. (A) Histological analysis of tubulointerstitial fibrosis in the renal cortex. Original magnification, ×400. (B) Quantitative analysis of tubulointerstitial fibrosis area. Scale bars represent 200 μm. [1]$p < .05$ vs. Vh, [2]$p < .05$ vs. Tac, [3]$p < .05$ vs. Tac + EM, [4]$p < .05$ vs. Tac + GG

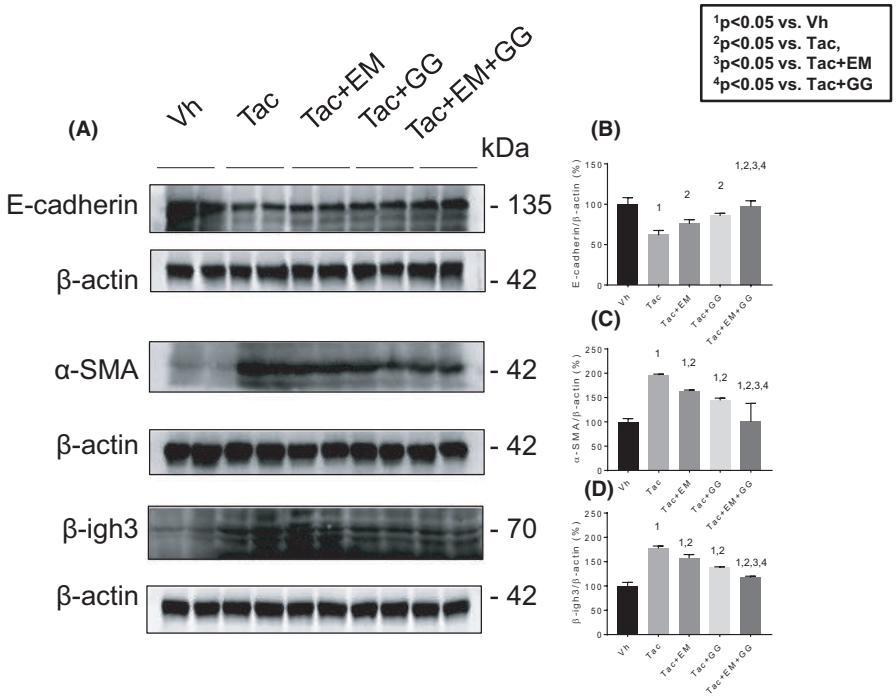

FIGURE 5 Effect of combined treatment of SGLT2 inhibitor and DPP4 inhibitor on the epithelial marker and fibrotic cytokine expression is in kidney. (A) Immunoblot analysis of (B) E-cadherin, (C) α-SMA, and (D) β-IGH3 in the renal cortex. β-actin protein expression was used for normalization. Results are expressed as mean ± SE and are representative of four independent experiments. [1]$p < .05$ vs. Vh, [2]$p < .05$ vs. Tac, [3]$p < .05$ vs. Tac + EM, [4]$p < .05$ vs. Tac + GG

measured via DCFA-fluorescence, significantly as compared with TAC (TAC 24.2 ± 0.8, TAC + EM 16.6 ± 2.6, TAC + Exd 20.0 ± 0.5, TAC + EM + Exd 4.0 ± 0.4 arbitrary units, $p < .05$). A similar finding was observed in cell death, as measured via PI staining; EM or Exd decreased the number of PI-positive cells significantly as compared with TAC alone (TAC 22.2 ± 0.1 vs. TAC + EM 19.8 ± 0.1, TAC + Exd

17.8 ± 0.3 arbitrary units, both $p < .05$), while combined treatment of both drugs further decreased the number of PI-positive cells when compared to each drug alone (12.9 ± 0.2 arbitrary units, all for $p < .05$).

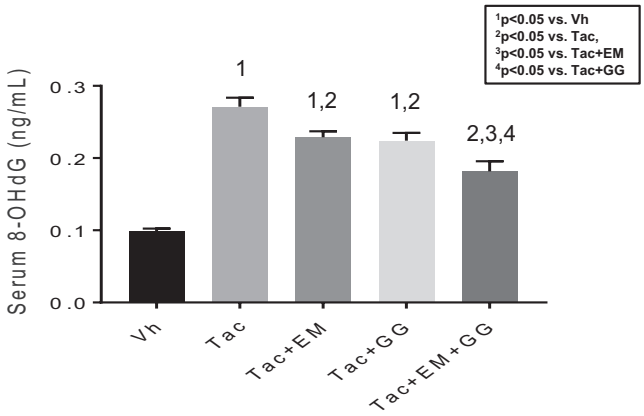

FIGURE 6 Effect of combined treatment with SGLT2 inhibitor and DPP4 inhibitor on serum 8-OHdG levels in Tac-induced DM. [1]$p < .05$ vs. Vh, [2]$p < .05$ vs. Tac, [3]$p < .05$ vs. Tac + EM, [4]$p < .05$ vs. Tac + GG

4 | DISCUSSION

The results presented here clearly show that combined treatment of SGLT2i and DPP4i is more effective in improving both TAC-induced DM and nephrotoxicity than each drug alone. Furthermore, dual inhibition of SGLT2i and DPP4i has a direct protective effect on TAC-induced renal injury and pancreatic islet injury. This finding provides a rational basis for the combined use of SGLT2i and DPP4i in patients with TAC-induced DM.

First, we evaluated whether the combination of SGLT2i and a DPP4i would provide better control of TAC-induced hyperglycemia compared to each drug alone. The IPGTT revealed that SGLT2i and DPP4i decreased blood sugar levels at 2 h by 21.0% and 22.3%, respectively, and combined treatment with SGLT2i and DPP4i further decreased blood glucose levels by 29.0%. In addition, SGLT2i or DPP4i treatment decreased HbA1C levels by 7.1% and 10.7%, respectively, and combined treatment further decreased HbA1C levels by 16.1%. Furthermore, the decreased plasma insulin levels and pancreatic islet size observed in the TAC group were increased with SGLT2i or DPP4i treatment and further increased with combined treatment. This finding confirms that a combination of SGLT2i and DPP4i provides better control of hyperglycemia, and improved preservation of pancreatic islet function in TAC-induced DM.

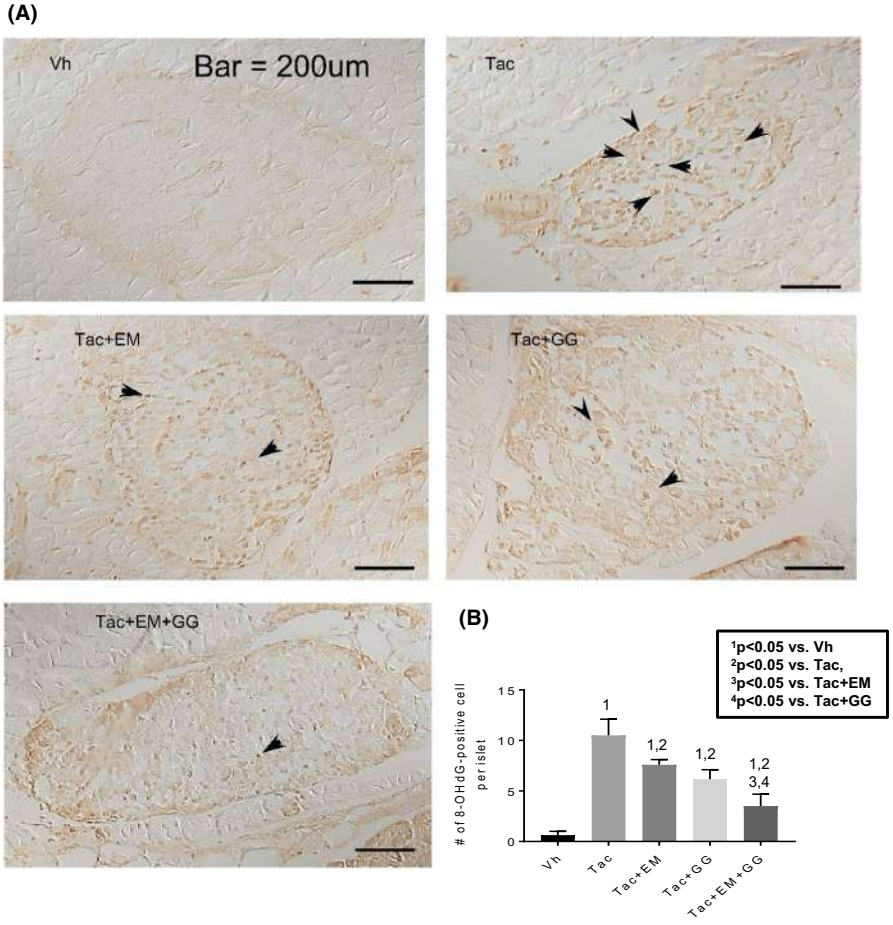

FIGURE 7 Immunohistochemistry of 8-OHdG in pancreatic tissue. (A) Immunohistochemistry of 8-OHdG in pancreatic tissues. Original magnification, ×400. (B) Quantitative analysis of 8-OHdG expression in pancreas section, ×400. Results are expressed as mean ± SE and are representative of four independent experiments. Scale bars represent 50 μm. [1]$p < .05$ vs. Vh, [2]$p < .05$ vs. Tac, [3]$p < .05$ vs. Tac + EM, [4]$p < .05$ vs. Tac + GG

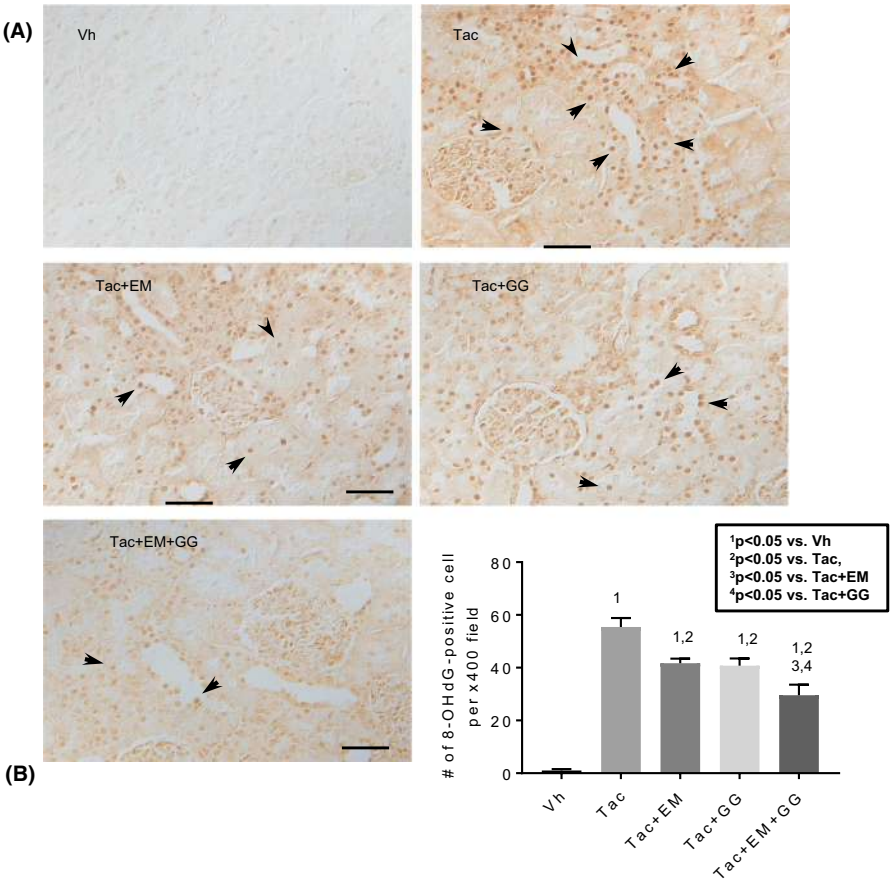

FIGURE 8 Immunohistochemistry of 8-OHdG in renal tissue. (A) Immunohistochemistry analysis of 8-OHdG in kidney tissues. Original magnification, ×400. (B) Quantitative analysis of 8-OHdG expression in kidney section. Results are expressed as mean ± SE and are representative of four independent experiments. [1]$p < .05$ vs. Vh, [2]$p < .05$ vs. Tac, [3]$p < .05$ vs. Tac + EM, [4]$p < .05$ vs. Tac + GG

Next, we evaluated whether the combination of SGLT2i and a DPP4i reduces TAC-induced renal injury. Our study revealed that both SGLT2i and DPP4i improved renal function and decreased interstitial fibrosis, and combined treatment further improved both parameters. At the molecular level, combined treatment with SGLT2i and DPP4i reversed the progression of epithelial-to-mesenchymal transition (as indicated by levels of E-cadherin expression) and decreased the levels of profibrotic molecules such as α-SMA and β-igh3, when compared with each drug alone. This finding was consistent with previous reports that SGLT2i and DPP4i prevent TAC-induced renal injury; our study extends this finding by indicating the combination of the two drugs can provide better protection. To define a protective mechanism, we evaluated the association between the development of DM and increased oxidative stress.[20] We previously reported that TAC-induced DM is associated with oxidative stress, and TAC-induced oxidative stress can be reduced by SGLT2i or DPP4i.[21] In this study, we evaluated the effect of combined treatment with SGLT2i and DPP4i on TAC-induced oxidative stress and found that combined treatment dramatically decreased the levels of an oxidative stress marker, 8-OHdG, in the serum, pancreatic islets, and kidney, compared with each drug alone. This finding may explain how hyperglycemia can be controlled and renal function improved by combining SGLT2 and a DPP4i in TAC-induced DM.

The results of our study showed that combined treatment with SGLT2i and DPP4i is more effective than treatment with either drug alone; however, it was unclear whether the combined effect direct or a secondary result of reduced glucose. Thus, we performed an in vitro study using INS-1 and HK-2 cells. The results of our study revealed that SGLT2i increased the viability in HK-2 but not in INS-1 cell lines. The reason for the different effects of SGLTi on INS-1 and HK-2 cells is unclear, but the interaction between SGLT2i and SGLT2 plays an important role in renal protective effect.[4] Our presumption may be supported by recent reports that insulin and ROS increase the expression of SGLT2 and glucose uptake in proximal tubular cells and SGLT-2 inhibitor protects against ischemia-reperfusion injury by inducing hypoxia-inducible factor 1.[22] Meanwhile, DPP4i increased viability in both cell lines. DPP4i exert their tissue-protective effect by enhancing the binding affinity of GLP-1 for GLP-1R. Actually, accumulating evidence has shown that the activation of GLP-1R signaling plays an important role in protecting cells (e.g., pancreatic islets and retinal cells) and organs (e.g., kidney and heart) against ischemic reperfusion injury by decreasing oxidative DNA damage and apoptosis.[23] Taken together, SGLT2 may play an important role in protecting renal tubular cells against hypoxic injuries under normal glucose conditions, but the lack of a direct protective effect of SGLT2i on pancreatic islets can be compensated for by the addition of DPP4i.[24,25] This may explain why the addition of DPP4i to SGLT2i is more effective in mitigating TAC-induced injury to pancreatic islets and the kidneys than SGLT2i alone.

Our study revealed that the combined treatment of SGLTi and DPP4i is effective in TAC-induced DM, but it is not certain that these

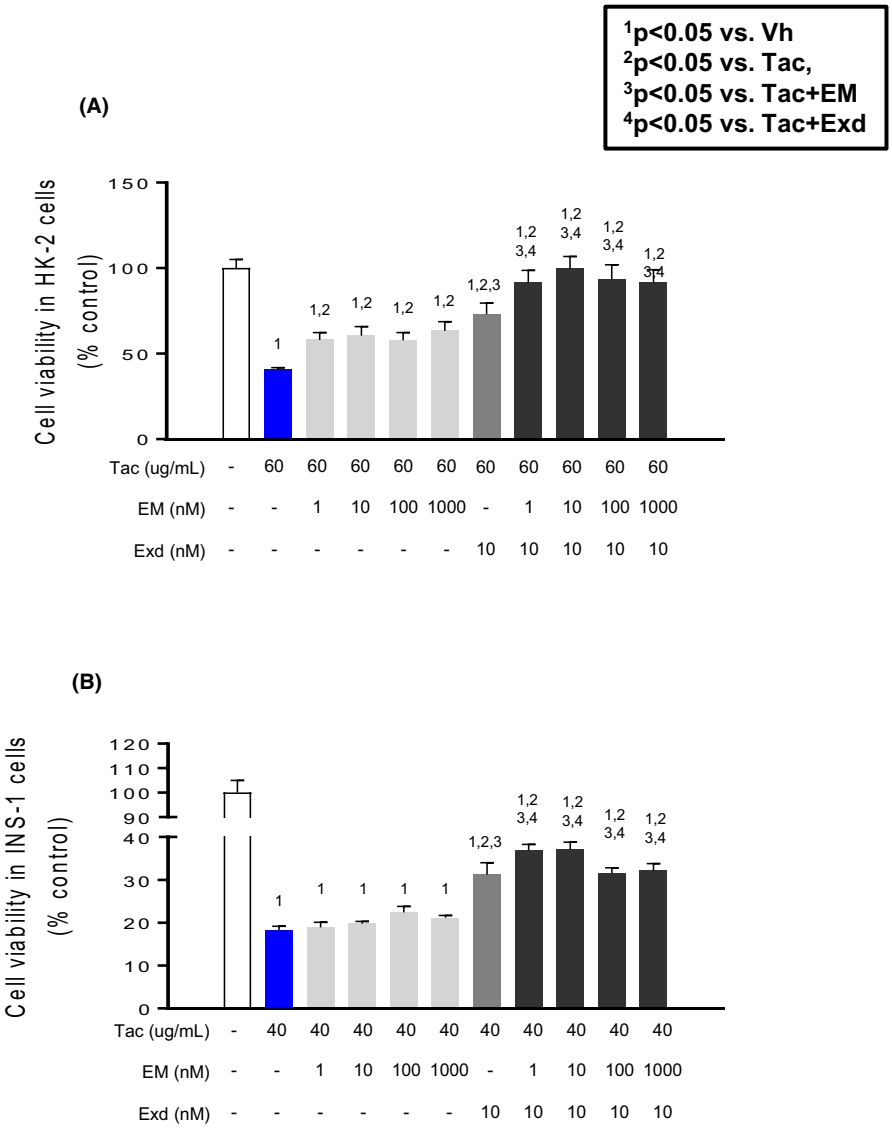

FIGURE 9 Cell viability of HK-2 proximal tubule cells and INS1 cells lines. (A) The viability of HK-2 cells and (B) INS-1 cells. [1]$p < .05$ vs. Vh, [2]$p < .05$ vs. Tac, [3]$p < .05$ vs. Tac + EM, [4]$p < .05$ vs. Tac + Exd

drugs are also effective in patients with pre-existing DM. Insulin resistance is increased in patients with pre-existing DM. Posttransplant diabetes mellitus resembles type 2 DM and reflects the interaction between pretransplant insulin resistance and calcineurin inhibitors. Previous studies have reported that tacrolimus increases pancreatic β cell toxicity in DM patients with insulin resistance.[26-29] Therefore, even in patients with pre-existing DM, the combination treatment of DPP4i and SGLT2i is expected to improve blood glucose control by tacrolimus. Another consideration is the clinical situation in that patients are treated with TAC and prednisone. In this study, we focused on TAC, but prednisone is also an important factor of PTDM. Thus, further study is needed on whether SGLTi or DPP4i are also effective in steroid-induced DM.

The mechanism of how these two drugs have synergistic effect on lowering hyperglycemia may be explained as complementary actions between two drugs. DPP4i reduces hyperglycemia by stimulating insulin secretion and inhibit glucagon secretion and SGLT2i reduces hyperglycemia by inhibiting glucose reabsorption at the proximal tubule. Therefore, we expect the synergistic effect of two drugs via different action mechanisms. Another consideration is that treatment with SGLT2i results in an increase in plasma glucagon concentrations, which was accompanied by a substantial increase in endogenous glucose production. This may offset approximately half of the glucose excreted in the urine. Thus, the addition of a DPP4i may enhance the glucose-lowering ability of SGLT2i by blocking the increase in endogenous glucose production and glucagon caused by SGLT2i.[30]

One may argue that the administration period of SGLT2i and DPP4i (3 weeks) is relatively short to evaluate the improvement of renal function and tissue damage, and more long-term treatment is needed in actual clinical practice. But, there is a discrepancy between animal study and clinical practice. Unlikely humans, high dose TAC is needed to induce renal injury because rats are resistant to TAC-induced renal injury. Thus, it is important to design animal study to get a positive result without drug-related death during the experiment. In our study, we determined the duration of the experiment as 6 weeks. With our protocol, we were able to get positive results of the drug on TAC-induced diabetes without any death in rats.

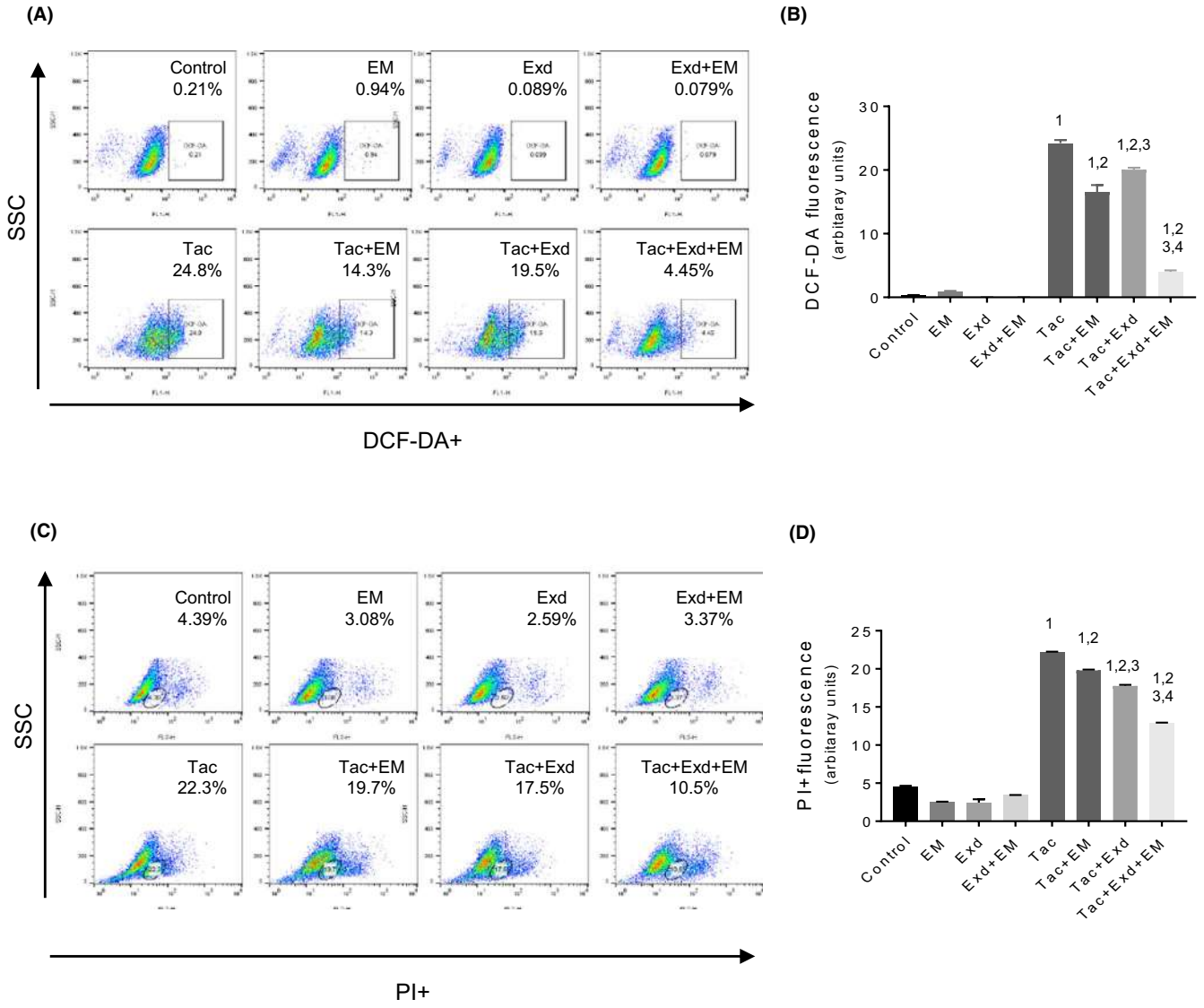

FIGURE 10 Flow cytometry of H2-DCFDA and PI-positive cell in HK-2 cells. (A) Intracellular ROS production using a flow cytometer. H2-DCFDA was used as a probe to evaluate intracellular ROS alterations (B) Quantitative analysis of ROS production. (C) Flow cytometry of PI. (D) Quantitative analysis of PI production. Results are expressed as mean ± SE and are representative of four independent experiments. $^{1}p < .05$ vs. Vh, $^{2}p < .05$ vs. Tac, $^{3}p < .05$ vs. Tac + EM, $^{4}p < .05$ vs. Tac + Exd

There may be concern about potential safety related to the use of these drugs. In this study, we performed assessments of safety and adverse-event profiles in terms of incidence of acute kidney injury (AKI), volume depletion with body weight, and hyperkalemia. In addition, we evaluated hypoglycemia, urinary infection, and pancreatitis. There was no difference in safety and adverse-event profiles except incidence of AKI among study groups. This finding suggested that combined treatment of these drugs did not cause additional serious side effects compared with each drug.

This current study has some limitations. First, there were no time-course and dose-response studies. Second, lack of insight into the exact cellular and molecular mechanisms that is responsible for these observations. In spite of these limitations, our study clearly demonstrates the benefits of combined treatment of is effective not

only in the control of hyperglycemia but also in protecting pancreatic islet and renal injury caused by TAC.

In conclusion, the management of PTDM with new anti-diabetic drugs requires clinical and experimental evidence. The results of our study provide experimental evidence that a combination of DPP4i and SGLT2i is effective not only in the control of hyperglycemia but also in protecting pancreatic islet and renal injury caused by TAC. This finding provides a strong rationale for combining SGLT2i and DPP4i in the treatment of TAC-induced DM in clinical practice; however, further clinical research is warranted.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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