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Synergistic Renoprotective Effects of Green Tea Extract and Gemigliptin against Tacrolimus-Induced Nephrotoxicity in Mice

Efectos Renoprotectores Sinérgicos del Extracto de Té Verde y Gemigliptina contra la Nefrotoxicidad Inducida por Tacrolimus en Ratones

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SUMMARY: Although tacrolimus (TAC) significantly reduces allograft rejection incidence in solid-organ transplantation, its long-term use is associated with an increased risk of TAC-induced nephrotoxicity. In this study, we investigated the renoprotective effects of green tea extract (GTE) with or without the dipeptidyl peptidase 4 inhibitor, gemigliptin, by assessing serum creatinine levels, the amount of proteinuria, and histopathology in TAC-induced nephrotoxicity. TAC-induced nephrotoxicity was induced by intraperitoneal TAC injection, GTE was administered via subcutaneous injection, and gemigliptin was administered orally. Mice with TAC-induced nephrotoxicity exhibited a significant increase in both serum creatinine levels and 24-hour urine protein. However, when treated with GTE via subcutaneous injection, mice showed a decrease in serum creatinine levels and the amount of proteinuria. When GTE was combined with gemigliptin, further renoprotective effects were observed in biochemical assessments, consistent with the attenuation of TAC-induced nephrotoxicity in histopathology. The expression of p53 protein was lower in the mice treated with the combination of GTE and gemigliptin compared to mice with TAC-induced nephrotoxicity. Our results demonstrate that the combination of GTE and gemigliptin treatment reveals synergistic renoprotective effects by decreasing the expression of p53 protein. These findings suggest that the combination of GTE and gemigliptin could potentially be used as a prophylactic or therapeutic strategy for TAC-induced nephrotoxicity.

KEY WORDS: Gemigliptin; Green tea extract; Nephrotoxicity; p53; Tacrolimus.

INTRODUCTION

Tacrolimus (TAC), a potent immunosuppressive medication, is widely used following solid-organ transplantation due to its highly effective reduction in the incidence of allograft rejection (Mayer *et al*., 1997; Webster *et al*., 2005). Nonetheless, long-term use of TAC can induce nephrotoxic effects, including renal fibrosis and tubular atrophy, which in turn contribute to chronic allograft dysfunction, resulting in a negative impact on graft survival (Shihab *et al*., 1997; Nankivell *et al*., 2016). Although the pathogenesis of TAC-induced nephrotoxicity remains unclear, it is suggested to involve a complex interplay of immunosuppressive actions, oxidative stress, and inflammation (Issa *et al*., 2013; Hoskov *et al*., 2014).

Green tea extract (GTE), derived from Camellia sinensis leaves, is rich in catechins, such as epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (Nain *et al*., 2022). Previous studies have demonstrated that these catechin compounds possess a wide range of beneficial properties, including anti-microbial (Reygaert, 2018), anti-oxidant (Yanagimoto *et al*., 2003) and anti-inflammatory effects (Musial *et al*., 2020). Furthermore, GTE has been shown to reduce the production of free radical species, suppress caspase-3 activation, and act as an antioxidant and free-radical scavenger, exhibiting protective effects against TAC-induced nephrotoxicity (Back *et al*., 2015; Oyouni *et al*., 2018).

Dipeptidyl peptidase 4 (DPP4) inhibitor (DPP4i) is an oral antidiabetic agent that lower glucose levels by inhibiting the degradation of incretins, such as glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide, by the enzyme DPP4 (Thornberry & Gallwitz, 2009). Several studies have showed that have potential

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renoprotective effects through reducing inflammation, fibrosis, and oxidative damage (Tanaka *et al*., 2014; Daza-Arnedo *et al*., 2021). In addition, DPP4i have been shown to provide renoprotective benefits in TAC-induced kidney injury through urinary exosome-derived miRNA profiling and anti-apoptotic effects (Huang *et al*., 2012; Cho *et al*., 2021). However, it remains unclear whether these protective effects can be further enhanced by combining DPP4i with GTE. This study aims to validate the synergistic renoprotective effects of GTE and DPP4i in a murine model of TAC-induced nephrotoxicity.

MATERIAL AND METHOD

Chemicals. TAC was purchased from Chong Kun Dang (Tacrobell™, Seoul, Korea). The gemigliptin (Gem), used as a DPP4i, was kindly supplied by LG Chemical (Gemiglo™, Seoul, Korea). GTE was prepared from a hot-water extract of green tea sourced from Boseong, Chonnam, Korea, following the previously reported method (Back *et al*., 2015).

Animals and treatments. All experimental procedures and care of animals were conducted following protocols approved by Chosun University's Institutional Animal Care and Use Committee (CIACUC2022-A0031).

Six-week-old male ICR mice (Samtaco Bio Korea; Daejeon, Korea) were divided into control, TAC, TAC + GTE, and TAC + GTE + Gem group (n = 6/each group). TAC-induced nephrotoxicity model (Back *et al*., 2015) was induced by administering TAC (3 mg/kg in 0.9 % normal saline, intraperitoneal injection on days 0, 3, 6, and 12), while control group received intraperitoneal injection of 0.9 % normal saline. The TAC + GTE group received 100 mg/kg of GTE subcutaneously every day. The TAC + GTE + Gem group received both 20 mg/kg of gemigliptin orally and 100 mg/kg of GTE subcutaneously (Fig. 1A). The dose of DPP4i (Lim *et al*., 2015) and GTE (Back *et al*., 2015) were followed as previously described. Mice were individually housed in metabolic cages for 24-hour urine collection on day 21. Mice were sacrificed on the 22nd day and blood samples were obtained.

Biochemistry analysis. Mouse serum creatinine level was measured using a biochemical autoanalyser (AVIDA 1650™, Bayer, Tarrytown, NY, USA). The amount of 24-hour urine protein was calculated using Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany).

Western blot analysis. Renal tissues were homogenized using ice-cold lysis buffer (pH 7.5) containing 137 mM NaCl, 20 mM Tris–HCl, 1 % Tween 20, 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail. Each sample was centrifuged at 14,000 rpm for 10 min at 4°C. The separated proteins were then loaded onto 10 % polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane. This membrane was subsequently incubated with primary antibodies against p53 and b-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After thorough washing, the membranes were incubated with a conjugated horseradish peroxidase anti-mouse secondary antibody (R&D Systems, Minneapolis, MN, USA) This secondary antibody was applied for 2 hours at room temperature. The detection of protein-antibody complexes involved using LumiGLO chemiluminescent substrate and visualizing the results through chemiluminescence, with the assistance of LabWorks software 4.0 (provided by UVP, Upland, CA, USA). The bands were quantified using the AzureSpot analysis software (version 14.2; Azure Biosystems, Inc., Dublin, CA, USA) and each result was normalized to b-actin.

Histopathologic analysis. For histopathologic examination, kidneys were fixed in 10 % buffered formalin, embedded in paraffin, and cut into 4 μm sections. The sections were then stained with hematoxylin and 4eosin (H&E).

Statistical analysis. The data are expressed as means ± SD of at least three independent experiments. Multiple comparisons between groups were performed by one-way ANOVA with Tukey's test for multiple comparison using Prism 9.5.1 (GraphPad). p values of <0.05 were considered significant.

RESULTS

Green tea extract and gemigliptin ameliorate TAC-induced nephrotoxicity with a synergistic effect. Mice with TAC-induced nephrotoxicity exhibited a significant increase in both serum creatinine levels and the 24-hour urine protein. Mice treated with GTE to TAC-induced nephrotoxicity showed a significant reduction in serum creatinine levels and proteinuria compared to TAC group. Interestingly, mice treated with the combination of GTE and DPP4i exhibited a significant reduction in serum creatinine concentration and 24-hour urine protein compared to TAC + GTE group (Figs. 1B and 1C).

Green tea extract and gemigliptin protect against kidney histopathological changes in TAC-induced nephrotoxicity. Histopathological analysis revealed that TAC-induced nephrotoxicity displayed vacuolization, tubular epithelial cell swelling, and necrosis (Fig. 2B) as

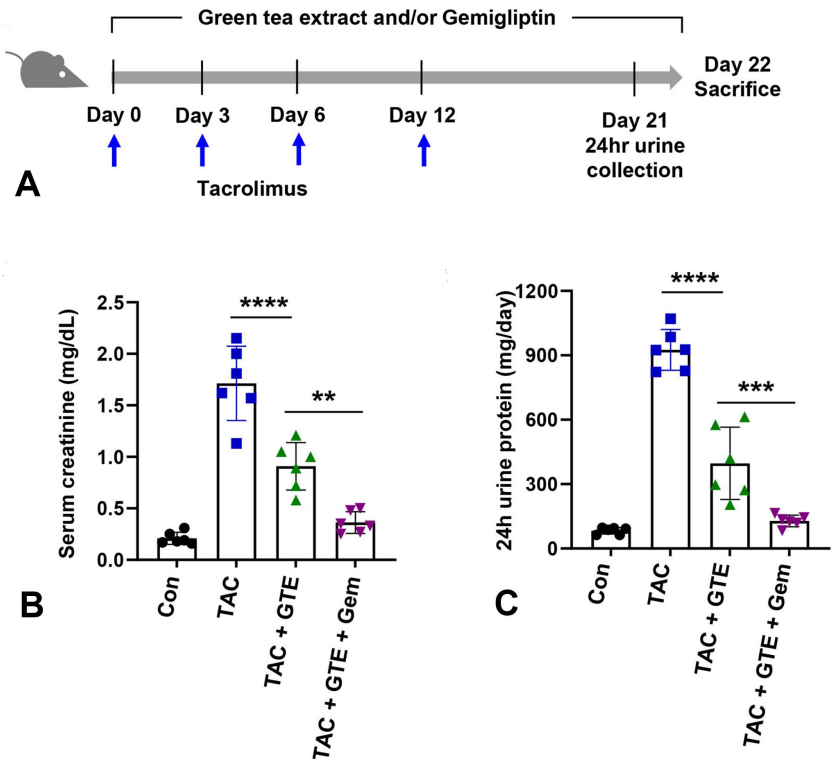

Fig. 1. Administration of green tea extract with or without gemigliptin ameliorate TAC-induced nephrotoxicity. (A) Experimental protocol: Tacrolimus (TAC)-induced nephrotoxicity was induced by administration of TAC (3 mg/kg in 0.9 % normal saline, intraperitoneal injection) on days 0, 3, 6, and 12. The green tea extract (GTE; 100 mg/kg, subcutaneous injection) and gemigliptin (Gem; 20 mg/kg, oral) were administered every day. Each mouse was individually housed in metabolic cages for 24-hour urine collection on day 21, and then sacrificed on the 22nd day and blood samples were obtained for further analysis. (B) Serum creatinine and (C) 24-hour proteinuria of each group in TAC-induced nephrotoxicity. Statistical analysis was performed using one-way ANOVA with Tukey's test for multiple comparison. **p < 0.01, ***p < 0.001, ****p < 0.0001. Data expressed as mean ± S.D. Con, control.

compared with control group (Fig. 2A). Treatment with GTE attenuated TAC-induced nephropathy (Fig. 2C), and the combination of GTE and gemigliptin resulted in no significant changes compared to the control group (Fig. 2d).

Green tea extract and gemigliptin reveal renoprotective effects through attenuating the p53 protein activation. To understand the mechanism by which the administration of GTE and gemigliptin attenuates TAC-induced nephrotoxicity, we performed a western blot analysis on the kidneys of experimental mice. The results of the Western blot analysis revealed that the activation of p53 protein was significantly increased in TAC-induced nephrotoxicity model. However, in mice treated with the combination of GTE and gemigliptin, there was a significant decrease in p53 protein expression as compared with TAC and TAC + GTE group, respectively (Fig. 3).

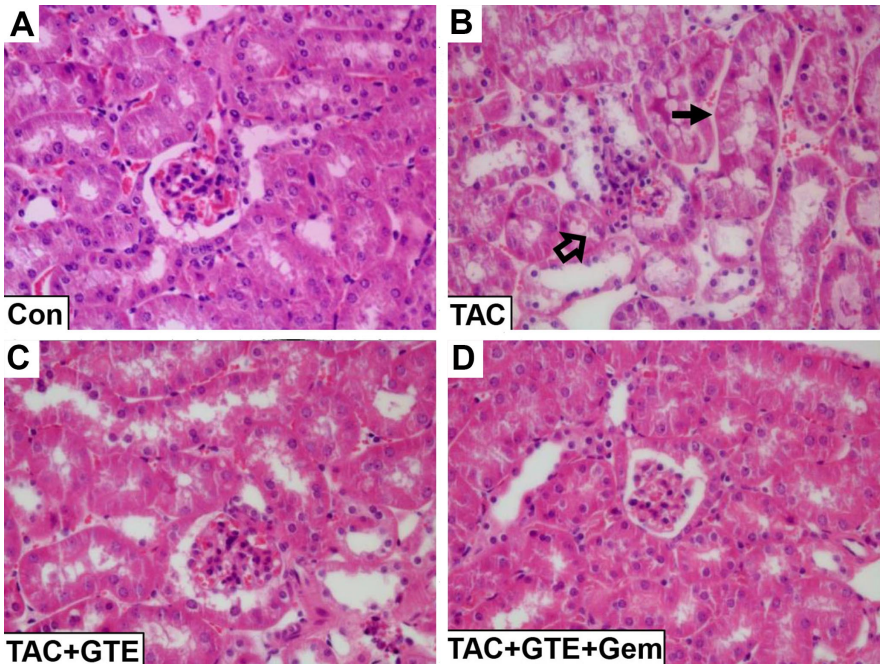

Fig. 2. Green tea extract and gemigliptin attenuate histopathological kidney changes in TAC-induced nephrotoxicity. Renal tissue from (A) control (Con), (B) tacrolimus (TAC)-induced nephrotoxicity, (C) TAC-induced nephropathy mice treated with green tea extract (GTE), and (D) TAC-induced nephropathy mice treated with combination of GTE and gemigliptin (Gem). Hematoxylin and Eosin stain (x 400). Arrow, loss of nucleus; Hollow arrow, vacuolization.

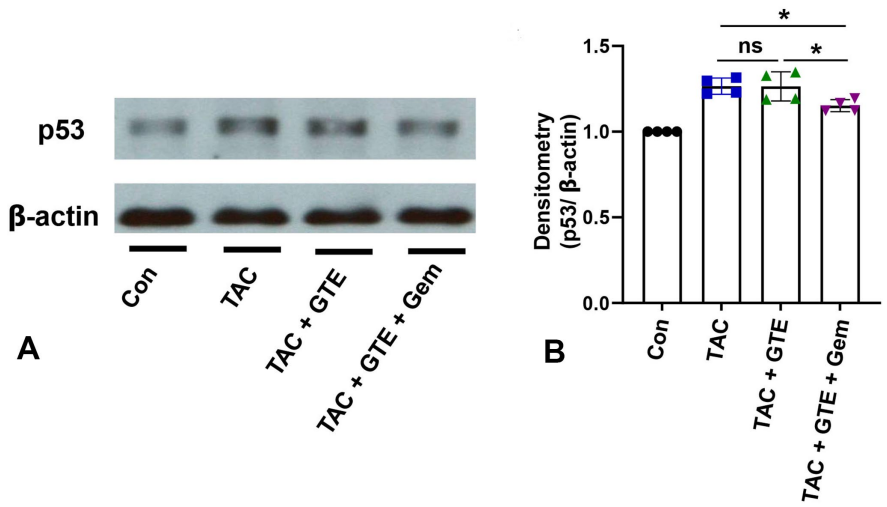

Fig. 3. Green tea extract and gemigliptin demonstrate renoprotective effects by reducing the expression of P53 protein. (A) Western blot analysis and (B) densitometry of p53 and b-actin expression in mice kidneys. Statistical analysis was performed using one-way ANOVA with Tukey's test for multiple comparison. ns, non-specific, *$p < 0.05$. Data expressed as mean ± S.D. Con, control; TAC, TAC-induced nephropathy; TAC+GTE, TAC-induced nephropathy mice treated with green tea extract (GTE); TAC+GTE+Gem, TAC-induced nephropathy mice treated with GTE and gemigliptin (Gem).

DISCUSSION

TAC, a calcineurin inhibitor (CNI), is widely used to prevent solid graft rejection by inhibiting T-cell proliferation through its binding to FK506 binding protein (Araya & Tasnif, 2023). However, TAC could cause nephrotoxicity with arteriolar hyalinization and thickening, vasoconstriction and ischemia, tubulointerstitial fibrosis, apoptosis, and atrophy (Randhawa *et al*., 1997). The renal histology of CNI nephrotoxicity is characterized by vacuolization of the proximal tubular epithelium, vascular injury, hyalinosis, and thrombotic microangiopathy, which are also observed in TAC-induced nephrotoxicity (Lusco *et al*., 2017). Previously, several agents, such as cilastatin, DPP4i, and aliskiren have demonstrated protective effects against TAC-induced nephrotoxicity (Al-Harbi *et al*., 2015; Lim *et al*., 2015; Luo *et al*., 2019). In this study, histopathological findings of TAC-induced nephrotoxicity, including tubular epithelial cell swelling and necrosis, decreased in mice treated with GTE compared to the TAC group (Figs. 2B and 2C). Furthermore, the combination of GTE and gemigliptin exhibited minimal histological changes of TAC-induced nephrotoxicity (Fig. 2D).

In this study, we observed that administration of GTE reduced serum creatinine levels and proteinuria in TAC-induced nephropathy mice compared to TAC group (Fig. 1B and 1c). Notably, the combination of GTE and gemigliptin demonstrated synergistic renoprotective effects compared to GTE treatment alone (Figs. 1B and 1C). The combined administration of these two drugs effectively ameliorated TAC-induced nephrotoxicity, potentially reducing the need for kidney biopsies in future patient applications. Proteinuria is a marker of kidney injury and a risk factor for progression to kidney disease, cardiovascular disease, and increased mortality (Hemmelgarn *et al*., 2010). Moreover, proteinuria is associated with reduced kidney allograft and patient survival after kidney transplantation (Roodnat *et al*., 2001; Jun *et al*., 2022). Therefore, the reduction of proteinuria plays a crucial role in slowing down the progression of kidney disease. Our study demonstrated that the combination of GTE and gemigliptin resulted in a reduction in the amount of proteinuria in a TAC-induced nephrotoxicity mouse model (Fig. 1c). In our experimental mice, the administration of GTE as well as the combination of GTE and gemigliptin effectively attenuated TAC-induced nephrotoxicity (Fig. 2.).

p53 is a transcription factor that regulates the expression of genes involved in apoptosis, cell cycle arrest, and DNA repair (Chen, 2016). The activation of p53 expression is known to be involved in cisplatin-induced nephrotoxicity, and inhibiting p53 has been shown to mitigate cisplatin-induced acute kidney injury (Jiang *et al*., 2004; Wei *et al*., 2007). The changes on p53 in TAC-induced nephrotoxicity in vivo have not been reported to the best of our knowledge. Our results showed that the expression of p53 protein was significantly elevated in the kidneys of TAC-induced nephrotoxicity mice. However, treatment with GTE with gemigliptin led to a significant reduction in p53 expression, although it still higher than control group (Fig. 3). These findings suggest that GTE and DPP4i may exert synergistic renoprotective effects by inhibiting p53 protein activation.

Taken together, we demonstrated that the combination treatment of GTE and gemigliptin exhibited synergistic renoprotective effects against TAC-induced nephrotoxicity in a mouse model. Further studies are needed to investigate whether the combination of GTE and DPP4i in clinical trials demonstrates renoprotective efficacy in kidney transplant recipients.

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RESUMEN: Aunque tacrolimus (TAC) reduce significativamente la incidencia de rechazo de aloinjertos en trasplantes de órganos sólidos, su uso a largo plazo se asocia con un mayor riesgo de nefrotoxicidad inducida por TAC. En este estudio, investigamos los efectos renoprotectores del extracto de té verde (GTE) con o sin el inhibidor de la dipeptidil peptidasa 4, gemigliptina, mediante la evaluación de los niveles de creatinina sérica, la cantidad de proteinuria y la histopatología en la nefrotoxicidad inducida por TAC. La nefrotoxicidad inducida por TAC se indujo mediante inyección intraperitoneal de TAC, el GTE se administró mediante inyección subcutánea y la gemigliptina se administró por vía oral. Los ratones con nefrotoxicidad inducida por TAC mostraron un aumento significativo tanto en los niveles de creatinina sérica como en la proteína en orina de 24 horas. Sin embargo, cuando se trataron con GTE mediante inyección subcutánea, los ratones mostraron una disminución en los niveles de creatinina sérica y en la cantidad de proteinuria. Cuando se combinó GTE con gemigliptina, se observaron efectos renoprotectores adicionales en las evaluaciones bioquímicas, lo que concuerda con la atenuación de la nefrotoxicidad inducida por TAC en histopatología. La expresión de la proteína p53 fue menor en los ratones tratados con la combinación de GTE y gemigliptina en comparación con los ratones con nefrotoxicidad inducida por TAC. Nuestros resultados demuestran que la combinación de tratamiento con GTE y gemigliptina revela efectos renoprotectores sinérgicos al disminuir la expresión de la proteína p53. Estos hallazgos sugieren que la combinación de GTE y gemigliptina podría usarse potencialmente como estrategia profiláctica o terapéutica para la nefrotoxicidad inducida por TAC.

PALABRAS CLAVE: Gemigliptina; Extracto de te verde; Nefrotoxicidad; p53; Tacrolimús.

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