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Clinical Transplantation

Is there any way to protect from tacrolimus-induced renal and pancreas injury?

Kaynar K, Ersoz S, Aliyazicioglu R, Uzun A, Ulusoy S, Al S, Ozkan G, Cansız M. Is there any way to protect from tacrolimus-induced renal and pancreas injury?

Abstract: Background: The aim of this study was to explore effects of erythropoietin and pentoxifylline in tacrolimus-induced pancreatic beta cell and renal injury in rats.
Methods: Rats in group I were given saline; rats in group II were injected with tacrolimus; rats in group III were received erythropoietin (Epo) and tacrolimus; while rats in group IV were injected pentoxifylline (Ptx) plus tacrolimus for nine d. On 10th day, blood and tissue samples were taken for biochemical and pathological evaluations.
Results: Tacrolimus-injected animals exhibited significant elevation in blood urea nitrogen (BUN), and serum BUN levels were improved in rats pretreated with Ptx. Significantly more apoptotic nuclei were observed in kidneys of tacrolimus group. In rats subjected to tacrolimus and pretreated with Epo, there was significant decrease in apoptotic nuclei staining than those in tacrolimus group. Blood trough levels of tacrolimus were significantly higher in erythropoietin-pretreated group, although same amount of tacrolimus was injected with other groups.
Conclusion: Results of our study demonstrated significant antiapoptotic effects of erythropoietin on renal tubules, increasing effect of erythropoietin on tacrolimus blood levels, and insignificant antioxidant effects of both erythropoietin and pentoxifylline on renal and pancreas tissues. Study with clinically greater tacrolimus levels may be useful to confirm these findings.

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Key words: apoptosis – erythropoietin – kidney injury – pancreas injury – pentoxifylline – rat model – tacrolimus

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Tacrolimus, as a calcineurin-inhibitor immunosuppressive drug, is widely used in organ transplantations; however, there are also several clinical side effects (such as nephrotoxicity, neurotoxicity, disturbances in glucose metabolism, gastrointestinal disturbances, and hypertension) associated with it. The occurence of diabetes after renal transplantation is recognized as one of the metabolic consequences of tacrolimus together with other risk factors like older age, obesity, genetic background, steroids, male gender, hepatitis C virus and cytomegalovirus infections, higher pretransplant glucose levels, and recipients of kidney from deceased donors (1). Diabetogenic effects of tacrolimus were associated with a decrease in insulin secretion rather than impairment of insulin receptor (2). To date, there has been no study of medication preventative treatments for tacrolimus-induced pancreas toxicity.

It has been well known that one of tacrolimus side effects is mediated by calcineurin inhibition. Chronic calcineurin inhibitor-induced nephropathy can ocur even on low-dose therapy, while acute renal damage is directly related to blood concentration (3). An early histological marker of tacrolimus-induced nephrotoxicity is vacuolization of proximal tubules; on the other hand, striped tubulointerstitial fibrosis, arteriolar hyalinosis, and tubular atrophy are noticed on long-term usage (4). One proposed explanation for these histological changes is calcium influx via dihydropyridine-sensitive calcium channels normally inhibited by calcineurin, leading to vasoconstriction, ischemia, and remodeling.

Erythropoietin (Epo) is the major regulator of proliferation and differentiation of erythroid progenitor cells through its antiapoptotic actions. In addition to its use in renal anemia, with increasing evidence, Epo has been reported to have broader functions independent of its effects on erythropoiesis. Erythropoietin has been found to have renoprotective effects in cisplatin and ischemia-induced renal failure (5). It was also reported that erythropoietin inhibits the caspase cascade and apoptotic cell death, stimulates tubular epithelial regeneration, and promotes renal function recovery in hypoxic or ischemic acute renal injury. Receptors of Epo are expressed in multiple tissues including glomerular, mesangial, and tubular epithelial cells in human, rat, and mouse kidneys, small bowel cells, myoblasts, neuronal cells, and pancreatic islet cells (6, 7). It was suggested that Epo might have antiapoptotic effects through increasing antiapoptotic genes in pancreatic islets (6).

Pentoxifylline (Ptx) is a non-selective inhibitor of cyclic-3′, 5′-phosphodiesterase, which is used in the treatment of peripheral vascular disease, exhibiting anti-inflammatory properties by inhibiting cytokine production, antioxidant properties by preventing release of superoxide and hydrogen peroxide ions, antithrombotic properties by inhibiting aggregation of erythrocytes and thrombocytes, and vasodilatator properties (8). Ptx administration was reported to ameliorate the histopathological alterations and the antioxidative enzyme levels in acute experimental pancreatitis (9). It has also clinically and experimentally exhibited the ability to exert renoprotective effects (10).

Therefore, we designed this study in rats to explore the effects of Epo and Ptx on tacrolimus-induced histopathological changes in kidney and pancreas and biological parameters, including malondialdehyde (MDA), blood urea nitrogen (BUN), and serum creatinine (Cr).

Material and methods

Female Sprague-Dawley rats (supplied by Surgery Research Center, Karadeniz Technical University, Trabzon, Turkey) that weighed 220–260 g and received standard diet and water *ad libitum* were used in a controlled room in which 12:12 h light/dark cycle was maintained. Research protocol was approved by and conducted according to the guidelines of the Karadeniz Technical University Animal Ethics Committee.

Grouping and experimental protocol

Twenty-eight rats were distributed equally into four groups. Rats in Group I were injected with 0.1 mL of saline intraperitoneally (i.p.); rats in group II were given daily intraperitoneal injections of tacrolimus (1 mg/kg; Prograf® 5 mg/mL ampule; Astellas Pharma, Dublin, Ireland) in a single dose; rats in group III were injected i.p. with 2000 IU/kg Epo (Eprex® 2000 IU/mL; Santa Farma, Istanbul, Turkey) plus 30 min later with tacrolimus (1 mg/kg) in a single intraperitoneal dose, while rats in group IV were injected i.p. with 80 mg/kg pentoxifylline plus 30 min later with tacrolimus at dose of 1 mg/kg (11–13). All injections were diluted with sterile saline.

Nine days of daily injections were performed to each group as written previously. Twenty-four hours after the last injection, rats were anaesthetized with intraperitoneal injection of ketamine hydrochloride (Ketalar®, 60 mg/kg; Eczacibasi, Turkey) and xylazine (8 mg/kg) (14). Via a midline incision, the abdominal cavity was exposed. Blood was obtained from trunk vessel, the left kidney was exposed and carefully separated from the adrenal gland, pancreas was harvested, and tissues were fixed in 10% formalin. The rats were killed under deep anesthesia with decapacitation after blood, renal, and pancreas tissue sampling.

Biochemical tests

Blood samples were centrifuged at 2000 g for 10 min to collect serum and stored −20°C for BUN and Cr levels. BUN and Cr were measured by a Roche Diagnostics Modular (DP) Analyzer using their own commercial kits (Roche Diagnostics GmbH, D-68298, Mannheim, Germany) for the assessment of kidney functions. Glucose was measured in the venous blood of the tail vein by Optium Xceed glucose meter (Abbott, Lake Forest, IL, USA), and it was regularly recorded every two d. Serum C-peptide levels were measured with enzyme immunoassay kits according to the

manufacturer's instructions (Catalog no: RSHAKRCP030R; Biovendor Laboratory Medicine Inc., Brno, Czech Republic). C-peptide levels were expressed as pg/mL. Determination of insulin was performed using enzyme immunoassay kits from Cayman Chemical Company/SPI-BIO (Ann Arbor, MI, USA, catalog no: A05105). Insulin levels were expressed as ng/mL. Blood tacrolimus trough levels were measured using ARCHITECT tacrolimus kits by Abbott ARCHITECT i2000SR analyzer via chemiluminescent microparticle immunoassay method.

MDA as a marker of lipid peroxidation product arises from the breakdown of lipid peroxyl radicals and oxidizes protein molecules; thus, it is a result as well as a cause of oxidative stress. Therefore, the extent of damage occurred to pancreas and kidney attributable to free radicals was assessed indirectly by measuring the MDA level. Tissues samples obtained from rats were kept at −80°C until biochemical analysis. Liver and pancreas tissue were weighted and homogenized in ice-cold 1.15% KCl (2% and 10% w/v, respectively). The homogenate was centrifuged at 2000 *g* for 10 min. Tissue MDA levels were determined by the method of Mihara and Uchiyama (15). Tetramethoxypropane was used as standard, and MDA levels were expressed as nanomole per gram wet tissue.

Morphological examination of pancreas and kidney

Renal and pancreas tissues were sectioned and fixed in 10% formaldehyde and embedded in paraffin for light microscopic assessment. Tissue sections were cut to 5 μm and stained with hematoxylin and eosin (H&E). Five coded slides from each group were examined using a light microscope (Olympus BX-50; Olympus Optical Co., Tokyo, Japan) from 10 randomly chosen areas per sample. Following specific features for pancreas damage were investigated: number of islets of Langerhans, mean size of islets, and islet features (cytoplasmic swelling, vacuolization, cell dropout, apoptosis, and nuclear atypia [karyomegaly, hyperchromasia, anisocytosis]). Renal histological findings: proximal tubular injury, interstitial inflammation, and scarring were searched. Findings indicative of tubular injury included cellular and intracellular vacuolization, tubular dilatation, and basement membrane thickening. Interstitial inflammation and fibrosis were defined by mononuclear infiltrates, edema in the interstitium, and by striping fibrosis with or without tubular atrophy and shrinkage (16). Apoptotic cell death in kidney sections was investigated using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling of DNA fragmentation (TUNEL) method (in Situ Cell Death Detection kit; Roche Diagnostics GmbH) (17). Staining procedure was performed in accordance with manufacturer's instructions. The percentage of TUNEL-positive cells in proximal and distal tubules was counted in four fields of 0.0025 cm^2 size by one investigator. The mean of numbers of labeled nuclei per fields was recorded.

Statistical analysis

We performed nonparametric analysis (because most of the variables were not normally distributed). Results were expressed as mean ± standard deviation (SD). A Kruskall–Wallis test was made for comparison of more than two groups, followed by a Mann–Whitney *U*-test for comparison of the two groups using computer-assisted program with SPSS for Windows 11.5 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered to be statistically significant.

Results

Effects of Epo and pentoxifylline on renal dysfunction mediated by tacrolimus

Animals that were given tacrolimus exhibited significant elevation in BUN (19.2 ± 2.9 mg/dL) compared with animals that were given saline (15.3 ± 1.2 mg/dL) ($p < 0.05$). Serum BUN levels were improved in rats pretreated with Ptx (14.6 ± 1.4 mg/dL). Compared with tacrolimus-treated animals, the administration of Epo decreased serum BUN levels (18.3 ± 1.5 mg/dL), but it was still significantly higher than animals in control group ($p < 0.05$) (Table 1). Although all values were in normal limits, serum creatinine levels were significantly higher in Epo + tacrolimus group (0.51 ± 0.04 mg/dL) than those in control group (0.37 ± 0.04 mg/dL) ($p < 0.05$), while they were not different between control group and Ptx pretreated (0.44 ± 0.05 mg/dL). Also no difference was noted between serum creatinine levels of control group and those in only tacrolimus given group (0.42 ± 0.04) (Table 1). Our histopathological studies showed normal findings in kidneys of the groups. However, it was shown in results of TUNEL (Fig. 1A–D) that significantly more apoptotic nuclei were observed in kidneys of tacrolimus group than those in control group (19.0 ± 6.6 and 7.2 ± 2.2 apoptotic nuclei/field, respectively) ($p < 0.05$). In rats subjected to tacrolimus and pretreated with Epo, there was no significant difference in apoptotic nuclei staining (10.3 ± 3.2 apoptotic nuclei/field) than those in control group

($p > 0.05$); on the other hand, in rats pretreated with Ptx (group IV), presence of apoptotic cells (18.2 ± 4.4 apoptotic nuclei/field) was significantly more than those in control group ($p < 0.05$) (Table 1). While tacrolimus (486 ± 105) and Epo +tacrolimus (477 ± 125) groups had insignificantly higher MDA levels than control (403 ± 30) and Ptx-pretreated (407 ± 48) groups, renal tissue MDA levels of groups were not significantly different (Table 1).

Effects of Epo and pentoxifylline on pancreas dysfunction mediated by tacrolimus

Serum glucose levels of groups were shown in Table 2. They were in normal range. Even though Epo-pretreated group had the lowest serum insulin concentrations, serum insulin concentrations were statistically similar between all groups after all treatments (1.40 ± 1.16 ng/mL; 1.36 ± 0.60 ng/mL; 0.74 ± 0.23 ng/mL; 0.92 ± 0.29 ng/mL; for control group; tacrolimus group; Epo+ tacrolimus group, and Ptx+tacrolimus group, respectively; $p > 0.05$; Table 1). Overall treatment did not affect C-peptide concentration of groups (Table 1, $p > 0.05$). Pancreatic damage scores were zero in all of the groups. Histological findings of all of the groups were normal. Counted mean islet numbers were 7.42 ± 3.73; 6.85 ± 2.85; 7.85 ± 2.96; 7.57 ± 3.10 in groups I; II; III; and IV, respectively ($p > 0.05$). Even though pancreatic tissue MDA levels of the groups were not significantly different,

Table 1. Biochemical and morphological test results of groups

	Control group	Tacrolimus group	Tacrolimus + Epo group	Tacrolimus + Ptx group
Creatinine levels of groups (mg/dL)	0.37 ± 0.04	0.42 ± 0.04	0.51 ± 0.04*	0.44 ± 0.05
BUN levels of groups (mg/dL)	15.3 ± 1.2	19.2 ± 2.9*	18.3 ± 1.5*	14.6 ± 1.4
Renal MDA levels of groups (nmol/g protein)	403 ± 30	486 ± 105	477 ± 125	407 ± 48
TUNEL + cells percentage of kidney tubule/field	7.2 ± 2.2	19.0 ± 6.6*	10.3 ± 3.2	18.2 ± 4.4*
Insulin levels (ng/mL)	1.40 ± 1.16	1.36 ± 0.60	0.74 ± 0.23	0.92 ± 0.29
Plasma C-peptide levels of groups (pg/mL)	307 ± 146	222 ± 40	239 ± 46	243 ± 116
Islet numbers of groups	7.4 ± 3.7	6.8 ± 2.8	7.8 ± 2.9	7.5 ± 3.1
Pancreatic MDA levels of groups (nmol/g protein)	88 ± 48	104 ± 40	163 ± 50	118 ± 50
Serum tacrolimus levels of the groups (ng/mL)		1.35 ± 0.60	3.87 ± 1.59[a]	1.70 ± 0.88

Data are presented as means of the values ± standard deviation (SD).
BUN, blood urea nitrogen; MDA, malondialdehyde.
[a]$p < 0.05$, vs. tacrolimus group and tacrolimus + Ptx group.
*$p < 0.05$, vs. control group.

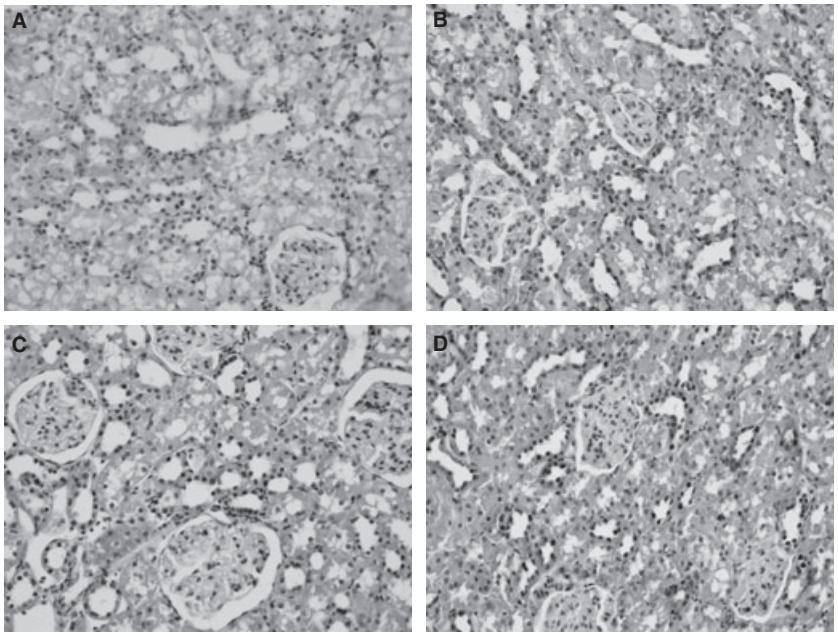


Fig. 1. Pictures of results of TUNEL (×40) in control group (A), tacrolimus group (B), Epo + tacrolimus group (C), and Ptx + tacrolimus group (D).

Table 2. Blood glucose levels (mg/dL) of groups on days 2, 4, 6, 8, and 10 (mean ± SD)

Groups	2nd day	4th day	6th day	8th day	10th day
Control group	88 ± 12	72 ± 3	75 ± 8	78 ± 6	72 ± 3
Tacrolimus group	69 ± 2	66 ± 11	72 ± 6	82 ± 18	66 ± 12
Tacrolimus + Epo group	84 ± 12	75 ± 7	56 ± 12	51 ± 2	75 ± 7
Tacrolimus + Ptx group	88 ± 3	94 ± 9	89 ± 5	86 ± 18	94 ± 9

MDA level of Epo-pretreated group was higher than those of other groups (Table 1). Tacrolimus levels were significantly higher in group III than those of the other groups ($p < 0.05$). The trough levels of tacrolimus on day 10 were 1.35 ± 0.60 ng/mL, 3.87 ± 1.59 ng/mL, and 1.70 ± 0.88 ng/mL in tacrolimus group, Epo + tacrolimus group, and Ptx + tacrolimus group, respectively (Table 1).

Discussion

Tacrolimus is currently widely used in transplant immunosuppression and the treatment of autoimmune diseases. Blockage of T-cell activation by calcineurin inhibition is also responsible for its most significant side effect nephrotoxicity. The deleterious effect of immunosuppression is unavoidable in renal transplantation. The precise mechanisms by which tacrolimus contribute to chronic allograft nephropathy over the long term are still far from clear. Even though reports on the effect of immunosuppression on apoptosis are conflicting, loss of renal parenchymal cells by apoptosis and involvement of caspase-3 have been reported to play important roles in progression of chronic allograft dysfunction (18). There are experiments suggesting oxygen free radicals as the underlying mechanism of tacrolimus-induced renal injury (19).

Our study demonstrated that tacrolimus application resulted in significantly higher BUN levels than saline injection in rats, and apoptotic cell percentage of kidney tubule was significantly higher in tacrolimus-injected group than those in control group. So we would have thought proapoptotic activity as the major renal injury mechanism of tacrolimus if erythropoietin pretreatment had not led to significantly increased BUN levels than saline injection and in the mean time inhibition of apoptosis to levels similar to control group. It was very well known that erythropoietin had antiapoptotic activity in kidney (20). We also found that erythropoietin had antiapoptotic activity in rat kidney tubules. However, we could not demonstrate antioxidant activity of Epo, which was previously reported (21). Epo-pretreated group had nearly similar MDA levels with tacrolimus group. But blood tacrolimus trough levels on 10th day were significantly higher in Epo-pretreated group than pentoxifylline-pretreated and only tacrolimus-injected groups. So with nearly three times higher tacrolimus levels, we would expect higher MDA levels in Epo-pretreated group than tacrolimus group. Instead, results of similar MDA levels make us think that Epo itself has significant antioxidant features. The higher tacrolimus levels of Epo-pretreated group may be because of effects of Epo on liver cytochrome P450 content. The relation between Epo synthesis and cytochrome P450 was already reported (22). Cell culture studies revealed that drugs which inhibit cytochrome P450 also lower Epo formation and drugs which induce cytochrome P450 also enhances synthesis of Epo. Likewise, in our study, three times higher tacrolimus levels were found in Epo-pretreated group than other groups even with the same amount of tacrolimus injection. So even with this higher blood tacrolimus levels, Epo pretreatment led to significant decrease in tacrolimus-induced renal apoptosis. The tacrolimus levels of group II were lower than therapeutic levels. This may be the underlying reason for failure of finding significant renal pathological lesions and significant increase in MDA levels. But even these low tacrolimus levels have led to significant increase in BUN levels and apoptotic cell nuclei.

A few studies have reported antiapoptotic effects of pentoxifylline on kidney tissue. In contrast, in our study, even with low blood tacrolimus levels, pentoxifylline had failed to prevent tacrolimus-induced renal tubular apoptosis. Still, pentoxifylline-pretreated group had similar BUN and creatinine levels with control group. MDA levels were lower in Ptx-pretreated animals than tacrolimus group. Even though there was no statistically significant difference, this antioxidant activity might play more important role than apoptosis in reflection of improved renal functions with final biochemical results.

In the literature, there are some experimental studies showing diabetogenic effetcs of tacrolimus, given daily oral doses of 10 mg/kg/d for two months and intraperitoneal doses of 0.1 mg/kg/d twice daily for nine d (23). A study by Malinowski et al. (24) failed to show diabetogenic effects of daily oral tacrolimus administered at 0.1, 0.5, and 1 mg/kg/d for 14 d. In our study, basal glucose levels (not after glucose loading) were in normal range after nine d of tacrolimus injection

at daily single i.p. dose of 1 mg/kg/d. Secretion of basal insulin and C-peptide was decreased in rats that received tacrolimus compared with those received saline. But this decrease in insulin and C-peptide secretion was not statistically significant and did not lead to basal hyperglycemia. This decrease in C-peptide levels might reflect slight and insignificant decrease in function of beta cells in a pancreatic islet. So tacrolimus application to rats with daily single intraperitoneal dose of 1 mg/kg/d for nine d did not influence pancreatic insulin and C-peptide production.

Pentoxifylline has known to have free radical scavenging properties and might be protective in tacrolimus-induced pancreas toxicity by increasing antioxidant defense mechanisms in addition to anti-inflammatory effects. However, in our study, as tacrolimus doses did not lead to pathological or biochemical endocrine pancreas damage, it was impossible to extract information about protective effects of Ptx from this injury. At least insignificant results made us think that no potentialization of tacrolimus-induced pancreatic injury occurred.

Effects of Epo on pancreas have not been much studied, but there are some reports showing inhibition of pancreatic beta-cell apoptosis of erythropoietin through its Epo receptor (6). In our study, even three times higher tacrolimus levels, we did not observe any decrease in islet numbers in Epo-pretreated group.

In conclusion, results of our study demonstrated significant antiapoptotic effects of combined Epo plus tacrolimus on renal tubules and also Epo-increased tacrolimus blood levels. There was insignificant antioxidant effects of both Epo and Ptx cotreatments on renal and pancreas tissues. There was no protective effect of Ptx on tacrolimus-induced renal toxicity. To more fully describe the tacrolimus, Epo, and Ptx treatment effects, a tacrolimus dose-ranging study with a greater range of tacrolimus concentrations may more fully reveal treatment effects on pancreas and renal injury.

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Author's contributions

Kubra Kaynar: participated in research design, participated in the performance of the research, participated in data analysis, participated in critical revision of the article, participated in drafting the article, participated in data collection; Safak Ersoz: participated in research design, participated in the drafting the article, participated in the performance of the research; Rezzan Aliyazicioglu: participated in research design, participated in the performance of the research, participated in the drafting the article; Aysegul Uzun: participated in research design, participated in the performance of the research, participated in data collection; Sukru Ulusoy: participated in research design, participated in critical revision of the article, participated in drafting the article; Sait Al: participated in research design, participated in the performance of the research, participated in data collection; Gulsum Ozkan: participated in data analysis, participated in data collection, participated in critical revision of the article; Muammer Cansiz: participated in data collection, participated in critical revision of the article, participated in data analysis.

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