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ORIGINAL ARTICLE

Renoprotective effect of Captopril on Tacrolimus-induced renotoxicity in mice

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ABSTRACT

Objective: To evaluate the renoprotective effect of captopril on tacrolimus-induced renal damage in mice.

Methods: This experimental study was conducted at Institute of Pathology and Diagnostic Medicine, Khyber Medical University, in collaboration with the Research Laboratory of Khyber Girls Medical College, Peshawar, over a period of six months. Twenty-one adult male albino mice were randomly assigned into three groups (n=7). Group 1 received 0.9% normal saline (control), Group 2 received tacrolimus (5 mg/kg/day orally), and Group 3 received tacrolimus (5 mg/kg/day) with captopril (10 mg/kg/day) orally for 21 days. On day 21, all mice were sacrificed after blood collection. Biochemical (serum urea, creatinine), morphological (absolute and relative kidney weights, length, width, and anteroposterior diameters), and histopathological parameters were assessed.

Results: Group 2 (tacrolimus only) demonstrated significant elevations in serum urea (33.85±9.83 mg/dl) and creatinine (0.25±0.05 mmol/l), along with marked histopathological alterations compared to Group 1 and Group 3 ($p<0.05$). Group 3 (tacrolimus+captopril) showed improvement in biochemical values (urea: 17.94±2.89 mg/dl; creatinine: 0.18±0.03 mmol/l) and attenuation of histopathological changes. Morphological variations included reduced absolute and relative kidney weights and changes in renal dimensions: shorter left kidney length (1.04±0.09 cm vs. 1.14±0.05 cm in controls) and increased right kidney anteroposterior diameter (0.52±0.04 cm vs. 0.44±0.05 cm in controls).

Conclusion: Captopril exhibited a renoprotective effect of more than 50% against tacrolimus-induced nephrotoxicity, reflected by improved biochemical, morphological, and histological parameters.

Keywords: Nephrotoxicity (MeSH); Dideoxyadenosine (MeSH); Oxidative Stress (MeSH); Histopathological (MeSH); Ichthyosis Bullosa of Siemens (MeSH); Biochemical (MeSH); Biochemical Phenomenon (MeSH); Tacrolimus (MeSH); Captopril (MeSH).

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INTRODUCTION

Tacrolimus, a calcineurin inhibitor, was familiarized clinically in the initial period of 1989 to substitute cyclosporine to treat patients with liver transplant who developed problems with obstinate organ dismissal or noxiousness due to drugs.[1] Earlier studies suggest that tacrolimus is utilized for prevention of organ rejection in heart, renal in addition to liver transplant. It is also used to treat many autoimmune diseases of the skin.[2] Kemper J, et al., showed that tacrolimus is linked with renotoxicity. Renotoxicity due to calcineurin inhibitors is a rare contributor to end-stage renal disease globally (3.2-4.8%). Among renal transplant recipients, the prevalence of calcineurin inhibitor nephrotoxicity spans from 76.4% after one year to 96.8% after 10 years post-transplant.[3] It has remained a major clinical issue. However, the main mechanism of nephropathy incited by tacrolimus is still unknown.[4] Studies, however, suggest that tacrolimus is able to produce reactive species of oxygen (ROS) by triggering nicotinamide adenine dinucleotide phosphate oxidase pathway, which leads to disruption of the antioxidant defense. In this way, reinforcing antioxidant protection in renal proximal tubule.[5] Angiotensin II plays major role in pathophysiology both, acute and chronic renotoxicity of tacrolimus. It releases aldosterone, stimulates transport through tubules, profibrogenic effects, proinflammatory effects, in addition to actions that prompt growth. Chronic calcineurin inhibitor renotoxicity as well as direct fibrosis of renal interstitium may occur due to activation of reactive oxygen species.[6] Studies have shown that tacrolimus lead to a surge in blood urea nitrogen (BUN) as well as levels of creatinine in serum. It reduces the endogenous levels of creatinine. It possesses an inverse proportion to the activity of plasma renin.[7] Sodium is expelled as the level of renin rises.[8] Biopsies of transplanted kidneys in patients treated with tacrolimus may result in morphological damage similar to other immunosuppressant agents used these days such as cyclosporine.[9]

Captopril is recognized as a first line drug to inhibit angiotensin converting enzyme. It opposes renin angiotensin aldosterone system (RAAS). RAAS regulates homeostasis, water besides electrolyte balance in the body.[10] Food and Drug Authority (FDA) recommends captopril for treatment of acute hypertensive crisis, dysfunction of left ventricle after myocardial infarction,

nephropathy in diabetes mellitus, Raynaud phenomenon, hypertension besides its use in congestive heart failure.[11,12] Inhibiting renin angiotensin system can delay the pathogenesis of renal failure. It can reduce renal injury by its antioxidant, antifibrotic, anti-inflammatory besides anti-apoptotic effects by decreasing nitric oxide, creatinine as well as urea levels.[13]

Earlier studies have revealed that tissues treated with captopril normalizes renal histology; it reduced degeneration and hypertrophy, dilation and/or atrophy of the tubules, proliferation of kidney cells in addition to normalizing of drug-incited kidney cell damage.[14] Since there is no former data that shows the renoprotective role of captopril against tacrolimus-induced renotoxicity. Therefore, tacrolimus was selected since it is amongst the exceedingly prescribed drug after tissue and allogenic organ is transplanted.[15] The present study was therefore; planned to investigate the protective effect of captopril on tacrolimus-induced renotoxicity in mice.

METHODS

The experimental study was conducted in animal house of Institute of Basic Medical Sciences (IBMS) Khyber Medical University (KMU), Peshawar. A sample of twenty-one adult male (Mus musculus) albino mice with an average weight of 25g-30g were purchased from Veterinary Research Institute (VRI), Peshawar. Sample size was calculated by Resource Equation Method.[16]

E=Total number of animals-Total number of groups

Where E is the degree of freedom and its value should lie between 10 and 20. So, E = 21-3=18

Ten additional mice were kept as a reserve using Attrition Formula.[17]

The experimental work was carried out in the Animal house of Institute of Basic Medical Sciences, Khyber Medical University, Peshawar. Approval was secured from Advanced Studies and Research board (ASRB) of Khyber Medical University, Peshawar (DIR/KMU-AS&RB/PE/001306) and the ethical committee of Khyber Medical University with the reference number of 6011/PGMED/KGMC. All the mice were housed in cages of the animal house of IBMS, KMU, Peshawar. The cages were labelled. The mice of each experimental group were numbered on their tails with a permanent marker. Simple randomized sampling technique was done to distribute the mice into three groups, each containing seven mice (n=7). The experiment adhered to the established animal safety protocols specified in the "Animal Bye-Laws" 2008 of the University of Malakand (Scientific Procedures Issue-1).[18] They were exposed to 12 hours/light 12 hours/dark cycle. Temperature was maintained in range of 20-25^{0}C to acclimatize the animals. The animals had free access to water. It was ensured to change the water every 24 hours. The mice were provided proper diet during the experimental period. The cages were cleaned regularly. Sawdust was changed daily. All these mice were weighed up by electronic animal weighing scale. The data was documented. All the animals were sacrificed on day 21. Terminal cardiac blood samples were collected to assess blood urea and creatinine. The kidneys of all the mice were dissected out after which these organs were preserved in a diluted solution of 10% formalin. Tissue processing was done in pathology laboratory of Khyber Girls Medical College, Peshawar. Later kidneys were divided into different sections to be examined under light microscope. To stain the tissues, hematoxylin and eosin stains were used.

Two distinct groups of drugs i.e. tacrolimus and captopril were used in this study.

Tacrolimus (Tacgraf) by CCL pharmaceuticals private limited was used for this study in a dose of 5 mg/kg body weight orally on the basis of previous studies.[19] Individualized doses were prepared for each mouse based on its weight by emptying capsules of 1mg strength into 10 ml distilled water, creating a milky suspension administered through oral gavage.

A pilot study was conducted to evaluate the renoprotective effect of captopril against tacrolimus in male albino mice using exponential test doses (0.5, 2.5, and 5 mg/kg). Captopril (Capoten) by GlaxoSmithKline pharmaceuticals (GSK) Pakistan limited was used for this study in a dose of 10 mg/kg body weight per oral was used on the basis of previous studies.[20,21] Other chemicals used in this study included distilled water for preparing drug solutions, 10% formaldehyde for preserving tissues according to the experimental protocol, chloroform for anesthetizing mice, and CMC for preparing the drug suspension.

Animals in group 1 (n=7) served as negative control group. They were administered 4ml per kg body weight of 0.9% normal saline per oral every twenty-four hours. Animals in group 2 (n=7) were given 5 mg/kg body weight of per oral tacrolimus every twenty-four hours. Animals in this group 3 (n=7) were given tacrolimus 5 mg per kg body weight per oral for twenty-one days. Captopril 10 mg per kg body weight dissolved in distilled water also given per oral for twenty-one days every 24 hours, simultaneously.

All the required mice were euthanized in succession by drop jar method in a gentle manner following the guidelines of American Veterinary Association Medical (AVMA).[22] Blood was obtained by cardiac puncture using a 1 mL syringe with a 22-23 gauge needle. Samples were taken to main clinical pathology laboratory of Khyber Teaching Hospital Peshawar. Renal biochemistry [serum urea (mg/dl) and serum creatinine (mmol/l)] was performed using autoanalyzer (Cobas® Autoanalyzer Machine, Roche diagnostics, Switzerland). Kidneys of all the mice were carefully separated, weighed (gms) and evaluated grossly for consistency. The information was documented. The tissues were transported to histopathology laboratory of KGMC, Peshawar following the required histopathology protocols.

After tissues were embedded in blocks, the various sections were stained with eosin and hematoxylin dyes (H and E). They were studied under light microscope to examine histopathological parameters. This included; dilation of capillaries, which also comprised their infiltration with erythrocytes; hypertrophy plus degeneration of renal tubules epithelia; renal glomeruli as well as epithelial cell enlargement in renal cortex besides

epithelial cells desquamation from the lumen in renal tubules.

Semi-quantitative scale[23] was applied for histopathological grading. The criterion was selected after discussion with three histopathologists of Peshawar.

Semi-quantitative scale reflective of the extent of histopathological change in renal tissue is as follows:

Normal=0, mild = <25%, moderate = 25-50% and severe= >50% of affected area. The score ranged from 0 to 3. Where 0 presented normal histopathology of the renal tissue. While 3 presented severe necrosis.

The data of the results attained was expressed in the form of mean value with standard deviation. Statistics software SPSS .20 was used for analysis. Comparison of biochemical markers between and within the groups was subjected to one-way ANOVA test. Post-Hoc Tukey was applied to the data. Kruskal-Wallis and Mann-Whitney test was applied for overall group comparison and pairwise significant comparisons of the histological parameters. $p < 0.05$ was considered as significant.

RESULTS

Comparison of blood urea and creatinine among Groups is expressed in Table I. The activity of all the mice in Group 1, 2 and 3 was noted both, before and after the experiment. It was observed that all the mice in Group 1, 2 and 3 were active before the experiment. They had sharp response to touch stimulus. The mice in all the groups had the same normal eating habits. While on day 21st, which marked the last day of the study, the mice in Group 2 were lethargic and inactive as compared to Group 1 and Group 3.They were not eating as normally as mice in Group 1 and were less responsive to touch stimuli as compared to mice in Group 1 and Group 3.

Absolute and relative bilateral kidneys weights were determined. This required determination of Mean body weight of mice first. The mean absolute body weights of mice in all the three experimental groups before and after

Table I: Effects of captopril on tacrolimus-induced changes in (a) renal function (b) absolute weights of bilateral kidneys (c) relative weights of bilateral kidneys

Variables		Mean±SD
Urea (mg/dl)	Control	20.6571± 2.32584
	Tacrolimus Treated	33.8571± 9.83495 [a]
	Tacrolimus+Captopril Treated	17.9429± 2.89589 [b]
Creatinine (mmol/l)	Control	0.1714± .01864
	Tacrolimus Treated	0.2571± .05589 [a]
	Tacrolimus+Captopril Treated	0.1886±.03237 [b]
Weight (g) Left Kidney	Control	0.2843±.02637
	Tacrolimus Treated	0.2357±.02070 [a]
	Tacrolimus+Captopril Treated	0.2157±.02225 [b]
Weight (g) Right Kidney	Control	0.2829±.02498
	Tacrolimus Treated	0.2443±.01512 [a]
	Tcrolimus+Captopril Treated	0.2414±.05080 [b]
Relative Weights of Left Kidneys	Control	1.0157±.09343
	Tacrolimus Treated	0.8457±.08904 [a]
	Tacrolimus+Captopril Treated	0.7429±.09759 [b]
Relative Weights of Right Kidney	Control	1.0243±.09289
	Tacrolimus Treated	0.8829±.05314 [a]
	Tacrolimus+Captopril Treated	0.8586±.18898 [b]
Length of Left Kidney	Control	1.143±.0535
	Tacrolimus Treated	1.100±.0577 [a]
	Tacrolimus+Captopril Treated	1.043±.0976 b
Length of Right Kidney	Control	1.100±.0000
	Tacrolimus Treated	0.929±.2289 [a]
	Tacrolimus+Captopril Treated	1.029±.0756 [b]
Width of Left Kidney	Control	0.586±.1069
	Tacrolimus Treated	0.557±.0535 [a]
	Tacrolimus+Captopril Treated	0.614±.0378 [b]
Width of Right Kidney	Control	0.586±.1069
	Tacrolimus Treated	0.743±.2820 [a]
	Tacrolimus+Captopril Treated	0.614±.0690 [b]
Antero-posteriordameter of Left Kidney	Control	0.443±.0535 [a]
	Tacrolimus Treated	0.514±.0690
	Tacrolimus+Captopril Treated	0.514±.0690 [b]
Antero-posterior diameter of Right Kidney	Control	0.443±.0535
	Tacrolimus Treated	0.471±.0488 [a]
	Tacrolimus+Captopril Treated	0.529±.0488 [b]

[a]The mean difference is significant at 0.05 level. The data are expressed as mean±SD (n=7). a=p<0.05% vs control group; b=p<0.05% vs toxic group. Analysis of variance (ANOVA) followed by Post Hoc Tukey-Kramer multicomparison Test

the experiment were calculated. Average weight calculated was 27.5 g. Effects of captopril and test drugs on absolute weights is shown in Table II.

Table I shows the absolute (organ) weights of bilateral kidneys expressed as mean and standard deviation and Relative weights of bilateral kidneys is calculated as ratio of absolute kidneys weight and body weight .

Relative Organ Weight = [Organ Weight / body weight] x 100.

Consistency of the mice kidneys after the experiment was checked by calculating and comparing the mean length, width and anteroposterior diameters (thickness) of bilateral kidneys [Figure 1].

Table III, Figure 2 and Figure 3 shows the protective effect of captopril on tacrolimus-induced renotoxic histomorphogical perspectives, semiquantitative scores of experimental groups, and histology of mice kidneys.

DISCUSSION

This study shows that Tacrolimus causes kidney damage in mice, seen through raised urea and creatinine levels, reduced kidney weight, and tissue injury. Captopril co-treatment reversed these effects, improving both renal function and histology. Biochemical recovery appeared earlier than tissue healing, confirming Captopril's protective role against Tacrolimus-induced nephrotoxicity.

Tacrolimus, a widely used calcineurin inhibitor, is associated with several adverse effects, including electrolyte disturbances, neurotoxicity, cosmetic changes, and most importantly, nephrotoxicity.[25] The precise mechanism underlying tacrolimus-induced renal injury remains unclear.[4] Previous studies have demonstrated that tacrolimus-related nephropathy is characterized by elevated serum urea and creatinine levels.[26] In line with these findings, tacrolimus-treated mice in the present study showed significant increases in blood urea and creatinine. For comparison, reference ranges for male mice are reported as blood urea 18-24 mg/dl (mean: 21) and creatinine 0.3-0.4 mg/dl (mean: 0.3).[27] The absence of significant differences between the control group (Group 1) and the captopril co-treated group (Group 3) ($p>0.05$) strongly supports the protective role of captopril in counteracting tacrolimus-induced biochemical renal damage, thus validating the study hypothesis. Tacrolimus exerts nephrotoxic effects primarily through direct cellular injury,[4] while its effect on glomerular filtration rate may be mitigated by the vasodilatory influence of captopril.[28]

Earlier reports suggested that tacrolimus or captopril therapy could alter body weight, potentially linked to tacrolimus-induced gastrointestinal adverse effects.[29] However, in the present study, neither tacrolimus nor captopril produced significant changes in mean body weight across experimental groups, which contrasts with previous findings.[30] Importantly, there is little evidence on the influence of body weight changes on the progression of renal disease.

Data on the effect of tacrolimus or captopril on individual organ weights are also scarce. In this study, tacrolimus-treated mice exhibited reduced absolute and relative weights of the left kidney, possibly due to vascular fibrosis from chronic drug exposure. The antifibrotic properties of captopril are thought to arise from its suppression of TGF and FGF expression and signaling,[31] which may prevent kidney atrophy and subsequent loss of weight.[32] Interestingly, mice co-treated with captopril showed further reductions in absolute and relative kidney weights compared to the tacrolimus-only group, while the right kidney weights remained unaffected. This contrasts with findings by Ntchapda F, et al., who observed attenuation of organ weight loss following captopril administration.[33] Current evidence regarding the effects of captopril on individual kidney weights remains limited. Another significant observation was the effect of tacrolimus on renal consistency. Tacrolimus-

Table II: Effects of Captopril on Tacrolimus-induced toxicity on absolute body weights of mice before and after experiment

Pairs		Mean±SD
Group 1 (Control)	Body Weight Before Experiment	27.35±1.74
	Body Weight After Experiment	27.50±1.80
Group 2 (Tacrolimus treated)	Body Weight Before Experiment	27.42±1.71
	Body Weight After Experiment	27.12±1.58
Group 3 (Tacrolimus+ Captopril treated)	Body Weight Before Experiment	27.49±1.50
	Body Weight After Experiment	27.51±1.64

The mean difference is significant at 0.05 level; The data are expressed as mean±SD (n=7); p>0.05%. Paired Sample t-test Sig (2-tailed) followed by Post Hoc Tukey Kramer Test.

Table III: Effects of Captopril on tacrolimus-induced renotoxic histomorphological perspectives

Histological Parameters	*Kruskal-Wallis Test	[a]Mann-Whitney Test Group 1 Vs Group 2	[b]Mann-Whitney Test Group 1 Vs Group 3	[c]Mann-Whitney Test Group 2 Vs Group 3
Hypertrophy and degeneration of renal tubules epithelia	0.000	0.000	0.000	0.317
Epithelial cells desquamation from the lumen in renal tubules	0.000	0.000	0.001	0.096
Renal glomeruli and epithelial cell enlargement in renal cortex	0.000	0.000	0.000	0.001
Dilation of capillaries and their infiltration with erythrocytes	0.002	0.002	0.002	0.593

*shows Asymp. Sig. p-values of all histological parameters. a,b,c shows 2-tailed p-values of all the histological parameters. Kruskal-Wallis Test followed by Mann-Whitney multiple comparison Test. *The mean difference is significant at $p<0.05$ level. Key: Group 1- Control, Group 2- tacrolimus treated, Group 3- tacrolimus+captopril treated

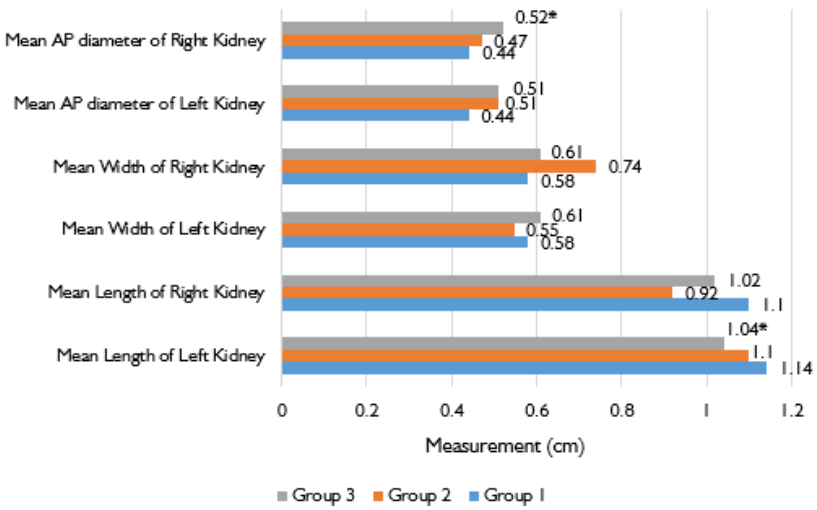

Figure 1: Effects of Captopril on tacrolimus-induced toxicity on Mean Consistency (Length, Width and AP diameter of Bilateral Mice Kidneys. AP-Anteroposterior) [*p<0.05% vs control group. Analysis of variance (ANOVA) followed by Post Hoc Tukey-Kramer multicomparison Test (Key: Group 1- Control, Group 2- tacrolimus treated, Group 3- tacrolimus+captopril treated)].

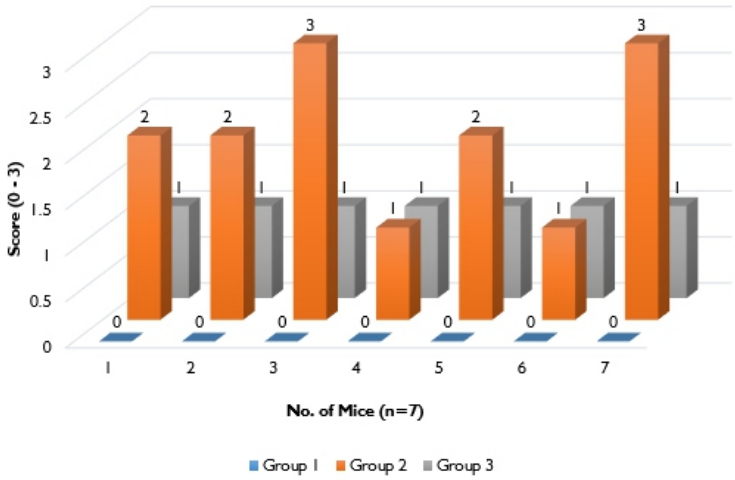

Figure 2: Semi-quantitative Score in mice kidneys of Experimental Groups exhibiting the Protective effect of Captopril on tacrolimus-induced Renotoxicity in mice. [Normal = 0, mild = <25%, moderate = 25-50% and severe = >50% of affected area. Semi-quantitive Scoring: Normal=0, mild=1. Moderate=2, Severe = 3 (Key: Group 1- Control, Group 2- tacrolimus treated, Group 3- tacrolimus+captopril treated)].

treated mice displayed alterations in renal morphology, while captopril co-treatment also influenced kidney consistency. Specifically, Group 3 mice demonstrated reduced left kidney length and increased right kidney anteroposterior diameter compared with controls. This may represent compensatory contralateral hypertrophy, measurement variability, or true structural changes. Drug-induced renal injury typically involves all nephron segments, including the glomeruli, tubules, interstitium, and vasculature, often secondary to renal arterial constriction.[34] In this study, tacrolimus-treated mice exhibited advanced histological alterations, notably epithelial cell desquamation in renal tubules, dilation of capillaries with erythrocyte infiltration, tubular degeneration, and glomerular hypertrophy.[9,35] Vascular dysfunction in tacrolimus toxicity is thought to result from activation of vasoconstrictor pathways, including the renin–angiotensin system, thromboxane, and endothelin, combined with reduced production of vasodilators such as nitric oxide and prostaglandins. Additionally, increased reactive oxygen species and direct juxtaglomerular stimulation by tacrolimus contribute to activation of angiotensin II, perpetuating a cycle of vascular damage.[35]

The current histological findings are consistent with those of Zhang LY, et al., who reported podocyte foot process effacement and ultrastructural injury in tacrolimus-treated rats.[4] Tubular hypertrophy and degeneration observed in our study may be attributed to isometric vacuolization of the cytoplasm ("osmotic nephrosis"), characterized by expansion of cytoplasmic reticulum, although the precise mechanism remains uncertain. These processes ultimately lead to desquamation and apoptosis of tubular epithelial cells.

Statistically significant changes were noted in all four histological parameters when comparing Group 1 (control) and Group 2 (tacrolimus). However, no significant differences were found between Groups 1 and 3, indicating that captopril ameliorated tacrolimus-induced renal injury. Semi-quantitative scoring further confirmed lower histological damage in Group 3 compared to Group 2, demonstrating the protective effect of captopril. These findings are in line with earlier studies in rat models.[7]

Interestingly, interim evaluation at day 15 showed that biochemical parameters had already improved in the captopril-treated group, while histological recovery lagged, suggesting that biochemical improvement precedes morphological repair.

Future Directions and Limitations of the study

Further research is warranted to confirm these findings in larger cohorts and with multiple dosing regimens, in order to establish dose–response relationships. Additional parameters such as total protein, albumin, blood pressure, glomerular filtration rate, and plasma renin activity could provide greater insight. Ultrastructural studies may also help elucidate the underlying mechanisms. Comparative studies with other renoprotective agents could

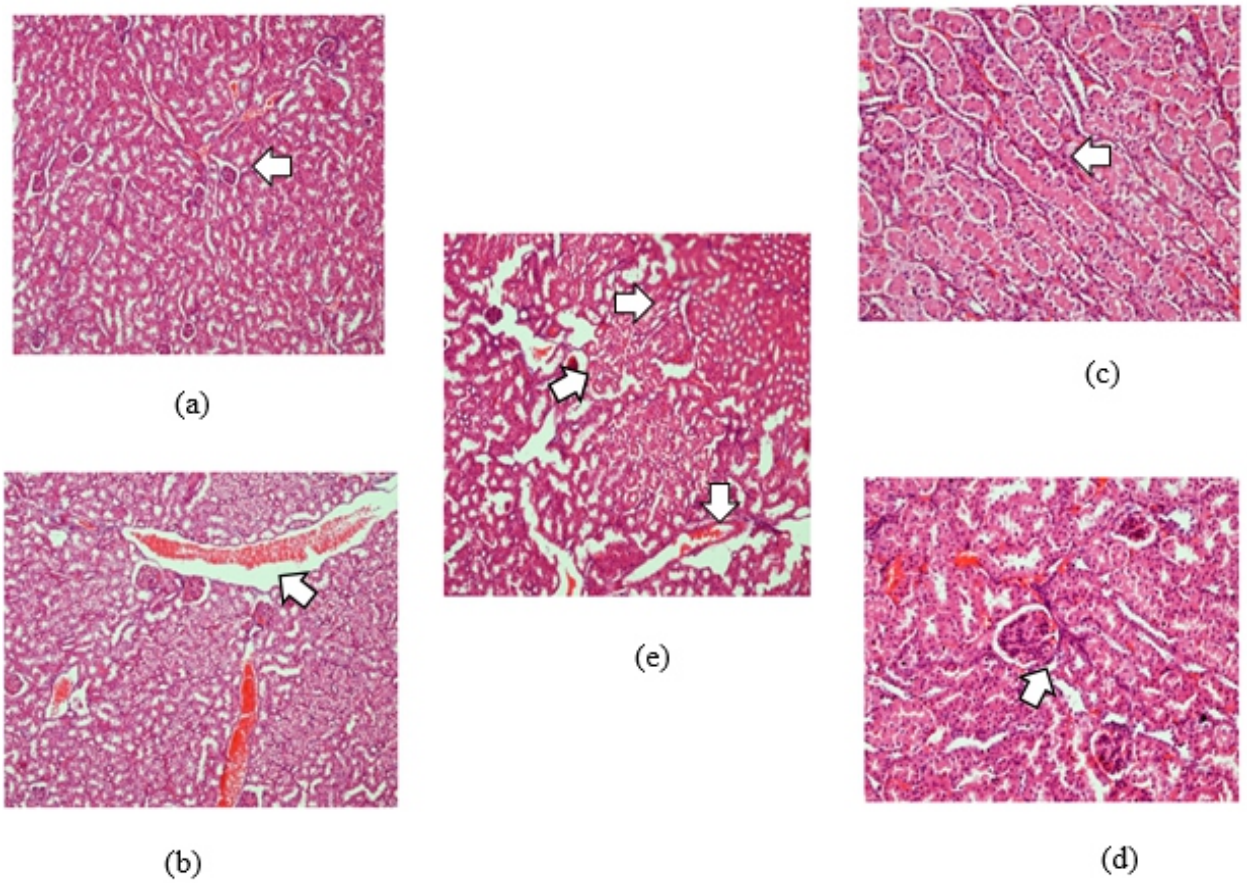

Figure 3: Effects of Captopril on Tacrolimus-induced Renotoxic changes on Histology of Mice Kidneys in various experimental groups. **(a)** Group 1: (4x Magnification, H&E; Normal) Renal glomeruli, tubular epithelial cells and capillaries with normal histology; **(b)** Group 2: (4x Magnification, H&E; tacrolimus) Dilated capillaries with infiltration of erythrocytes; **(c)** Group 2: (10x Magnification H&E; tacrolimus) Degeneration of tubular epithelial cells with desquamation of the epithelial cells; **(d)** Group 2: (10x Magnification, H&E; tacrolimus) Renal glomeruli enlargement with degeneration of tubular epithelial cells; (e) Group 3: (10x Magnification, H&E; tacrolimus with captopril) Improvement in Renal glomeruli size and capillaries diameter, degeneration of tubular epithelial cells with desquamation as compared to tacrolimus alone. Key: Group 1- Control, Group 2- tacrolimus treated, Group 3-tacrolimus +captopril treated.

broaden therapeutic options. Development of a combined tacrolimus–captopril formulation may also be explored.

The main limitations of this study include its small sample size and the use of a single captopril dose. Nevertheless, the data strongly suggest that captopril can mitigate tacrolimus-induced nephrotoxicity, with protective effects evident at both biochemical and histological levels.

CONCLUSION

Overall, our findings confirm the nephrotoxic potential of tacrolimus, evident in both biochemical and histological changes. Captopril co-treatment provided significant renoprotection, improving both biochemical and histological parameters, with biochemical recovery occurring earlier. These results highlight the potential clinical value of captopril in reducing tacrolimus-induced nephrotoxicity.

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AUTHORS' CONTRIBUTION

The Following authors have made substantial contributions to the manuscript as under:

SI: Conception and study design, acquisition, analysis and interpretation of data, drafting the manuscript, approval of the final version to be published

AHK, NA, NS: Conception and study design, analysis and interpretation of data, drafting the manuscript, critical review, approval of the final version to be published

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RF: Analysis and interpretation of data, drafting the manuscript, critical review, approval of the final version to be published

SU: Analysis and interpretation of data, critical review, approval of the final version to be published

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

Authors declared no conflict of interest, whether financial or otherwise, that could influence the integrity, objectivity, or validity of their research work.

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