PROTECTIVE EFFECT OF *SPINACEA OLERACEA* EXTRACT ON CYCLOSPORINE A INDUCED NEPHROTOXICITY IN MALE ALBINO RATS

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Abstract

Background and Objective: Cyclosporine A (CsA), is a powerful immunosuppressant that has revolutionised the field of transplantation. CsA causes nephrotoxicity which is characterised by arteriolar hyalinization, shrunken glomeruli and stripped tubular necrosis. Spinach (Spinacea oleracea) has an antioxidant and anti-mutagenic potential. The present study was designed to observe the effects of spinach extract against CsA induced histological changes in kidneys.

Methods: Twenty eight rats (6 – 8 weeks) were divided into 4 groups; A, B, C and D. Group A served as control and was given 2 ml/kg of olive oil intraperitoneally for 7 days. Group B was given Cyclosporine A, 20 mg/kg dissolved in 2 ml of olive oil intraperitoneally for 7 days. Group C was given 0.5 ml of 1% n-hexane extract of Spinacea oleracea for 14 days concomitantly with Cyclosporine A, 20 mg/kg dissolved in 2 ml of olive oil intraperitoneally from day 7 to 14. Group D was given 0.5 ml of 1% n-hexane extract of Spinacea oleracea orally for 14 days.

Results: Histological sections of kidneys of CsA treated rats showed extensive vacuolation, necrosis and loss of brush border in tubular cells, peri-tubular infiltration and vascular congestion. There was decrease in mean glomerular diameter. Concomitant administration of Spinacea Oleracea (SO) extract with CsA, significantly ameliorated all these renal lesions.

Conclusion: The findings of this study suggest that SO extract has protective effect against the CsA induced nephrotoxicity.

Key words: Cyclosporine A(CsA), Nephrotoxicity, Spinacea Oleracea(SO).

INTRODUCTION

Cyclosporine A (CsA) is a powerful immunosuppressant recommended as first line therapy after immunomodulatory disorders and transplantation.[1] CsA use is associated with nephrotoxicity. Almost all the patients using this drug presented with kidney lesions.[2]

CsA is a highly lipophilic endecapeptide. It is metabolized by the enzymes of cytochrome P450 system and is eliminated in bile and urine.[3,4] CsA is used for the treatment of arthritis, cirrhosis, cardiac hypertrophy and neurodegeneration.[5-7] It has also been investigated to prevent tumour progression.[8]

Cyclosporine exerts immunosuppression by inhibiting the calcineurin and kinases and inducing the increased expression of transforming growth factor β.[19] CsA reduced the activity of immune system after binding with cyclophilin A, which is present on T-lymphocytes and is a part of mitochondrial permeability transition pore.[10]

Acute CsA induced nephrotoxicity is characterised by arteriolar hyalinisation and stripped tubular necrosis and interstitial infilteration. Haematoxyline and eosin sections stained sections of CsA treated group showed vacuolation and necrosis of cortical tubules. Vascular congestion and fibrosis was observed in interstitium. These changes were accompanied with disrupted brush borders and inclusion bodies in cytoplasm of tubular cells on PAS stained sections.[11-13] Glomeruli were shrunken and there was increase in Bowman's space.[14] On histopathological grading, extensive vascular congestion, hyaline casts, cortical vacuolar degeneration and interstitial inflammation were demonstrated.[15] CsA had deleterious effects on foetal kidneys of albino mice.[16]

CsA induces histological damage in kidney through various mechanisms such as; over stimulation of renin angiotensin system, oxidative stress leading to lipid peroxidation and disruption of cell membranes and increase of vascular resistance resulting in renal ischaemia.[17-19]

Spinacea Oleracea (SO) has free radical scavenging properties.[20] SO significantly reduced lipid peroxidation and offered protection against radiation induced injury.[21] SO had ameliorative effect on carbon tetra chloride induced liver damage by optimizing the action of oxidative enzymes.[22]

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The toxic effect of CsA on different organs had been well studied but protective effect of *Spinacea oleracea* extract on CsA induced nephrotoxicity has not been seen. Consequently, the aim of the present study was to evaluate the effect of *Spinacea oleracea* on histological structure of rat kidney treated with cyclosporine A.

ANIMALS AND METHODS

Experimental Protocol

It was an experimental study, carried out at the Experimental and Research Laboratories and Anatomy department of UHS, Lahore.

Male *Wistar* Albino rats 28 in number, 6 – 8 weeks old and weighing 150-175 g, were procured from the animal house of University of Health Sciences, Lahore. They were examined to exclude any evidence of disease before beginning of experiment. The rats were housed with controlled temperature of 22 ± 3˚C; humidity was set at 55 ± 5% and light and dark cycles of 12 hours each. Normal chow and tap water ad libitum were allowed to the animals. Rats were kept for a week for acclimatization.

Chemicals

Cyclosporine A (Neoral) was purchased from Novartis (Switzerland) and dissolved in olive oil as reported by Mohamadin et al.[23]

The n-hexane extract of *Spinacea oleracea* was prepared in Pharmacology lab, UHS, Lahore. Fresh leaves were purchased from local market, dried, chopped, soaked in n-hexane and condensed by rotary vacuum evaporation. The oil obtained after condensation was dissolved in olive oil.[24]

Animal Groups

Rats were divided randomly into four groups of seven rats each (Table 1).

Group A served as control and was given 2 ml/kg/day of olive oil intra-peritoneally as a vehicle for 7 days and sacrificed on 8th day.

Group B was given Cyclosporine A, 20 mg/kg/day dissolved in 2 ml of olive oil intra-peritoneally for 7 days and sacrificed on 8th day.

Group C was given 0.5 ml of 1% n-hexane extract of *Spinacea oleracea* orally for 14 days and Cyclosporine A, 20 mg/kg dissolved in 2 ml of olive oil intra-peritoneally for 7th to 14th day. This group was sacrificed on 15th day.

Group D was given 0.5 ml of 1% n-hexane extract of *Spinacea oleracea* orally for 14 days. This group was sacrificed on 15th day.

The previous studies were used to determine the dose and duration of the study.[14,24] Doses were adjusted according to the body weight of the animals.

At the end of experimental period rats were euthanized and both kidneys were removed for histological examination.

Histological Examination

The kidneys of each rat were removed, fixed in 10% formalin solution, processed in the automatic tissue processor and embedded in paraffin. Paraffin blocks were cut into sections of 5 µm thickness using automatic rotary microtome. These sections were then stained with haematoxylin and eosin. The slides were examined with light microscope using X10 and X40 magnification. These sections were assessed for tubular necrosis, vacuolation, casts, interstitial congestion and inflammation. Diameter and number of glomeruli were also recorded.

Statistical Analysis

SPSS version 20.0 (Statistical Package for Social Sciences) was used to analyze the data. Mean ± SD was calculated for quantitative variables. One way ANOVA/ Kruskal-Wallis test was applied to compare means of quantitative parameters among the groups. Post Hoc Tukey Test was applied for group wise comparison. Fisher exact test/chi square was used to observe association among qualitative variables.

Table 1: *Showing experimental grouping of animals and experimental intervention.*

Groups	*Intervention and Dosage*	*Route of Administration*	*Duration of Administration*	*Day of Sacrifice*
Group A	Olive oil 2 ml/kg/day	Intraperitoneal	Day 1 – 7 (Week 1)	8
Group B	Cyclosporine A 20 mg/kg/day dissolved in 2 ml of olive oil	Intraperitoneal	Day 1 – 7 (Week 1)	8
Group C	*Spinacea oleracea* extract 0.5 ml	Orally	Day 1 – 14 (Week 1+ Week 2)	15
	Cyclosporine A 20 mg/kg dissolved in 2 ml of olive oil	Intraperitoneal	Day 7 – 14 (Week 2)	
Group D	*Spinacea oleracea* extract 0.5 ml	Orally	Day 1 – 14 (Week 1+ Week 2)	15

RESULTS

Effect of SO on Kidney Morphology

Kidney sections from group Aanimals showed clearly demarcated cortex and medulla. Cortex of the renal sections of group A contained renal corpuscles which appeared as dense round structures comprising of glomerulus housed within double layered Bowman's capsule lined by simple flattened epithelial cells with flattened nuclei. A narrow urinary space was separating the inner visceral and outer parietal layer of the Bowman's capsule. Glomerular capillaries were cut in longitudinal, oblique and transverse section. Cortical tubules occupied most of the renal parenchyma. Proximal tubules showed dark eosinophilic cuboidal cells and prominent brush border. Cells of distal convoluted tubules were lightly stained, more nuclei present per section as the cells are smaller and microvilli were absent (Fig. 1).

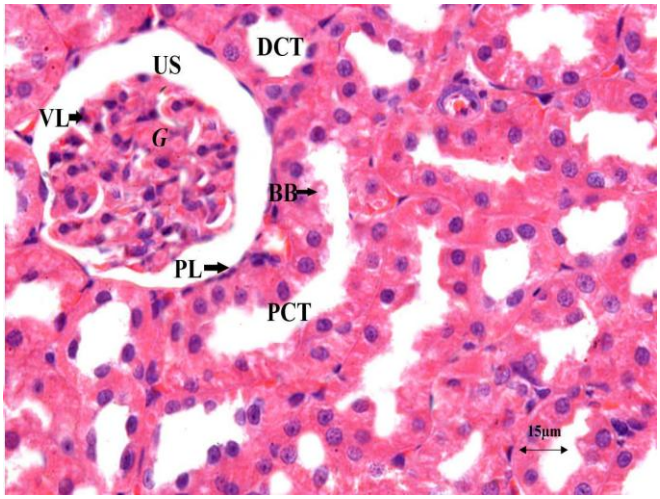

Fig. 1: *Photomicrograph of cortex of kidney from Control group A showing normal glomerulus (G), having capillaries lined by endothelium and mesangial cells, enclosed by visceral layer (VL) and parietal layers (PL) of Bowman's capsule having urinary space (US) in between. Proximal convoluted tubule (PCT) lined with cuboidal epithelium having distinct brush border (BB) and distal convoluted tubule (DCT) with simple cuboidal epithelium. H&E. X400.*

In group B there were strips of necrosed tubules with complete obliteration of lumen and increased eosinophilia. Tubular epithelial cells showed marked necrosis (p-value = 0.001) and vacuolation (Table 2). Glomeruli were sclerosed with increase in urinary space. Interstitium showed marked inflammation and vascular congestion (p-value = 0.001) (Fig. 2).

In cortical section of group C there was restoration of normal histology however; some of the tubules exhibited vacuolation and desquamation of epithelial cells but renal architecture was well preserved (Fig. 3). While sections of group D were nearly comparable to control group A (Fig. 4).

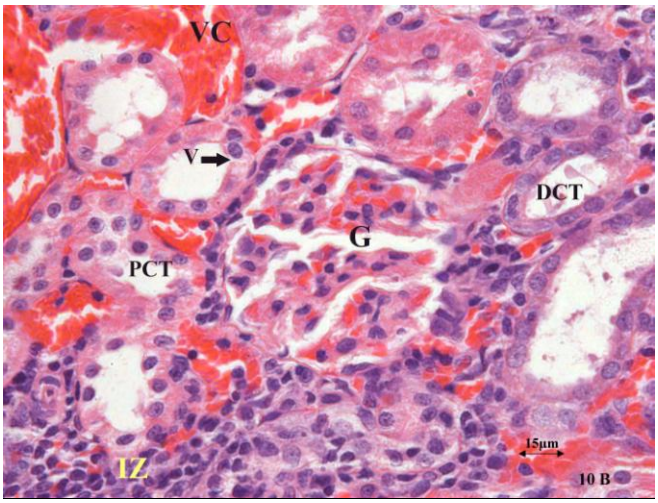

Fig. 2: *Photomicrograph of cortex of kidney from group B showing glomerulus (G), proximal convoluted tubule (PCT) lined with cuboidal epithelium having vacuolation (V), disrupted cell boundaries and distal convoluted tubule (DCT) with degenerating cuboidal cells. Interstitium showing inflammatory zone (IZ) and vascular congestion (VC). H&E. X400.*

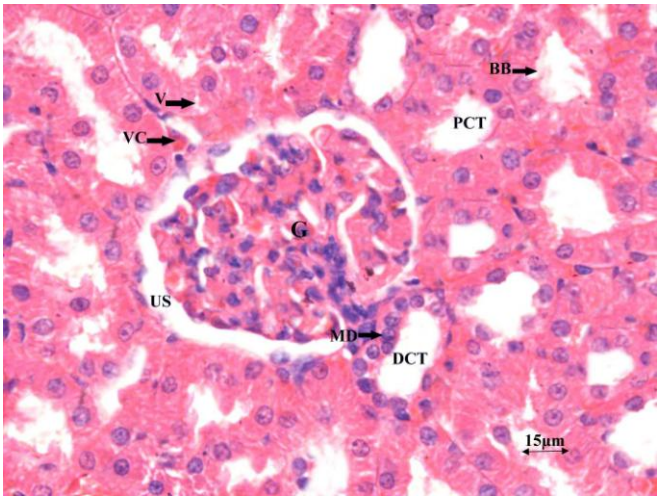

Fig. 3: *Photomicrograph of cortex of kidney from Prophylactic group C showing glomerulus (G) enclosed by visceral and parietal layers of Bowman's capsule having urinary space (US) in between. Proximal convoluted tubule (PCT) lined with cuboidal epithetlium having distinct brush border (BB), vacuolations (V) present in a few cells. Distal convoluted tubule (DCT) with simple cuboidal epithelium forming macula densa (MD) near vascular pole of renal corpuscle. Slight vascular congestion (VC) still observable. H&E. X400.*

Effect of SO on Glomerular Diameter and Number:

Significant difference was observed in the value of

mean diameter of glomerulus (p-value = 0.001*) when compared between groups A, B, C and D. Post Hoc Tukey test showed that the value of mean diameter of glomerulus of groups A and D were comparable while it was significantly reduced in CsA treated group B when compared with groups A and D. Glomerular diameter in group C was also significantly decreased (Table 3).

The difference was not statistically significant when mean number of glomeruli/mm² (p-value = 0.167) in the kidney section of the rats of groups A, B, C and D were compared with one another respectively (Table 3).

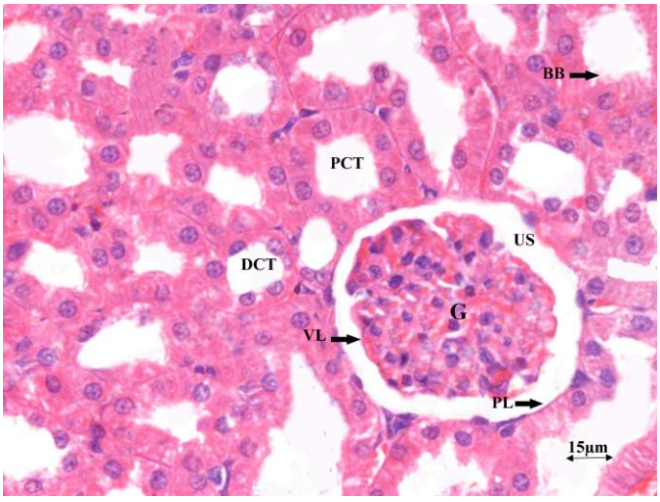

Fig. 4: *Photomicrograph of cortex of kidney from group D showing normal glomerulus (G) enclosed by visceral (VL) and parietal layer (PL) of Bowman's capsule having urinary space (US) in between. Proximal convoluted tubule (PCT) lined with cuboidal epithelium having prominent brush border (BB) and distal convoluted tubule (DCT) with simple cuboidal epithelium. H&E. X400.*

DISCUSSION

In the current study CsA treatment caused a discernable damage to kidney structure involving tubules, interstitium and arterioles. Considerable degree of vacuolation, ill-defined brush borders and necrosis in tubular epithelial cells was observed in H & E stained sections of CsA treated group. Similar findings were reported in the previous studies.[12,13,25] They attributed these findings to increased expression of genes involved in apoptosis.

Several studies are available to explain the underlying mechanisms involved in CsA induced nephrotoxicity like renal hypoxia mediated by arteriolopathy due to activation of intrarenal renin angiotensin system (RAAS).[26] Arteriolopathy results in obliteration of arterioles and necrosis + sclerosis of the corresponding glomerulus and associated parenchyma. Prolonged ischemia due to vasoconstriction also cause cellular atrophy.[27] CsA activated caspases, the enzymes of programmed cell death, ultimately lead to renal cell death.[28] Renal and hepatic stores of glutathione are depleted by CsA causing lipid peroxidation,[29] it also upregulated renal cytochrome p450 system producing imbalance in radox status.[30]

Table 2: *Showing comparison of percentage of tubular necrosis among groups.*

Epithelial Necrosis	*Percentage of Necrosis*				
	Group A n = 7	*Group B n = 7*	*Group C n = 7*	*Group D n = 7*	*Total*
Present	0 (0.0%)	7 (100%)	1 (14.3%)	0 (0.0%)	8 (28.6%)
Absent	7 (100%)	0 (0.0%)	6 (85.7%)	7 (100%)	20 (71.4%)
Total	7	7	7	7	28
Fisher Exact Test = 20.81				p-value □ 0.001*	

*p-value ≤ 0.05 is considered to be statistically significant.

Table 3: *Showing comparison of mean diameter and number of glomerulus among groups A, B, C and D.*

Parameter	*Group A Mean ± SD*	*Group B Mean ± SD*	*Group C Mean ± SD*	*Group D Mean ± SD*	*p-value*
Diameter of Glomeruli (µm)	86.5 ± 2.16	66.71 ± 4.93	68.25 ± 6.97	93.57 ± 6.34	0.001*
Number of Glomeruli/mm²	8.5 ± 1.58	10.28 ± 1.55	9.43 ± 2.22	8.28 ± 1.70	0.167

*p-value ≤ 0.05 is considered to be statistically significant.

Luminal casts in cortical tubules were observed in CsA treated group. In acute tubular necrosis (ATN) tubular lumen is obstructed by casts formed by aggregation of polymers of "Tamm-Horsfall protein" and cellular debris; this protein is normally secreted by tubular cells as monomer but in ATN it become evident in histological sections.[31]

In the present study marked peritubular vascular

congestion and inflammation was observed in CsA treated group, it is in accord to the previous studies.[32,33] Tubular cells after injury were activated, released cytokines which attracted the inflammatory cells in the interstitium,[34] this explains the presence of interstitial inflammation seen in present study. CsA through RAAS activation also leads to increased production of angiotensin II, which upregulates receptors which contribute to glomerular and interstitial inflammation.[35]

The difference in the values of glomerular diameter in the current study was statistically significant (p value = 0.001); in CsA treated group diameter of the glomerulus was significantly reduced when compared to groups A and D, this is in agreement with previous studies on CsA induced nephropathy which reported sclerosed and shrunken glomeruli with widening of Bowman's space.[11] These glomerular changes are possibly due to altered renal hemodynamics caused by CsA[36] which decreased glomerular filtration rate by causing increased afferent arteriolar resistance due to vasoconstriction.[37] High levels of reactive oxygen species in endothelial and mesangial cells were also demonstrated.[38] Moreover, vascular necrosis and glomerular thrombosis by CsA has also been reported.[39]

Co-administration of *Spinacea oleracea* extract partially reverted CsA induced histological damage by scavenging the free radicals as reported by previous studies.[18] Restoration of all these parameter indicate the protection offered by *Spinacea oleracea* extract on CsA induced nephropathy, conferred by its active ingredients such as p-coumaric acid and quercetin.

From this experiment it is ***concluded*** that spinacea oleracea extract partially reverts CSA induced nephrotoxicity and ultimately resects in restoration of the disturbed histologic parameters.

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Author's Contribution

MA: Design of the work, Data collection, Data analysis and interpretation, drafting the article. MT: Concept and design of the work, Critical revision of the article, Final approval before publication. KPL: Data interpretation, Critical revision of the article.

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