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Impact of thymoquinone on cyclosporine A pharmacokinetics and toxicity in rodents

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Keywords
cyclosporine; drug interaction; hyperglycaemia; nephrotoxicity; pharmacokinetics; thymoquinone

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Abstract

Objectives Cyclosporine A (CsA) is an immunosupprsant drug used to prevent graft rejection and in the treatment of several autoimmune diseases. Thyomquinone (TQ), a bioactive component of *Nigella sativa*, has strong antioxidant properties and has been used in prevention of many toxicities, hence its protective effect and pharmacokinetic interactions with CsA was investigated in this study.

Methods For bioavailability study, the rats were divided into four groups: TQ (PO, 10 mg/kg) was given alone for 7 days, then TQ plus CsA for another 5 days, CsA was given by two routes (po) and (IP) in a dose of 10 mg/kg 1 h after administration of TQ. Blood samples were taken at the 12th day at specified times, CsA level was determined by immune assays. The protective effect of TQ was studied. Blood samples for lab investigations and histopathology were taken at the 28th day.

Key findings Thyomquinone reduced the bioavailability of oral CsA by around 32% ($P > 0.05$). However, bioavailability of IP administered CsA was not affected. Chronic administration of CsA increased concentrations of fasting glucose and Cystatin C and produced marked s kidney alteration of parenchyma which was reversed by concomitant administration of TQ.

Conclusions A potential drug interaction between TQ and CsA, which may reduced its oral bioavailability. Independently TQ caused significant attenuation of CsA induced renal toxicity and diabetogenic effect.

Introduction

Cyclosporine A (CsA) is a calcineurin inhibitor (CNI) which remains essential components of immunosuppressant regimens in many transplant centres and frequently used for management of immune diseases. Mechanism of action of CsA involves selective suppression of T-lymphocyte. Long term use of CsA is associated with several significance adverse effects.[1]

Multiple drug interactions are also likely to occur in transplant patients due to polypharmacy, dietary supplements and alternative herbal medication/supplements. Most of the drug or diet interactions involve induction or inhibition of cytochrome P-450 system (cyt P450) or P-glycoprotein pump which may have serious consequences such as toxicity, failure of immunosuppressive action or even rejection of the transplanted organ.[2] CsA is extensively metabolized by hepatic cytochrome system (CYP450 3A 4/5), which is subjected to considerable interindividual variation and drug interaction.[3] CsA has a narrow therapeutic window; insufficient dosing may pose the increased risk of organ rejection, whereas overdose is associated with toxicity.[4]

Although being an effective drug, there are some drawbacks of using CsA. Chronic use of CsA is known to have side effects most common of them being nephrotoxicity and hyperglycaemia.[2] CsA also causes abnormal glucose homeostasis by decreasing pancreatic insulin release and increasing peripheral insulin resistance resulting in development of hyperglycaemia.[5,6]

Thymoquinone (2-Isoprop l-5-methylbenzo-1,4-quinone) (TQ), is the bioactive component of volatile oil of black seed, *Nigella sativa*. Many researches demonstrated that TQ has nephroprotective, anti-hyperglycemic, hypolpidmic, anti-inflammatory, anti-neoplastic and hepatorotective activity.[7,8] TQ was reported to inhibit CYP3A, an enzyme which is responsible for metabolism of majority of drugs.[9] This might lead to herb-drug interaction, which calls for its clinical assessment.

Since CsA is also metabolized by cytochrome system (hepatic CYP3A), this study was designed to elucidate the effect of TQ on bioavailability of CsA and to independently evaluate its protective effect against CsA-induced nepherotoxicty and hyperglycaemia.

Materials and Methods

Cyclosporine A oral (colourless clear solution, 100 mg/ml) and injectable forms (colourless oily solution, 50 mg/ml) were purchased from NOVARTIS Pharmaceuticals (Australia), Pty Limited, 54 Waterloo Road, Australia.

Thyomquinone powder (99%) was purchased from Sigma-Aldrich chemicals (St. Louis, MO, USA).

Preparation of cyclosporine A vehicle

- Oral CsA: Half ml of oral cyclosporine solution (as supplied) was added to 4.5 ml of distilled water, mixed well, a clear solution was obtained (10 mg/ml) and was used immediately.
- IP CsA: One ml of injectable forms (50 mg/ml) was added to 4 ml of dextrose 5%, mixed well, a clear solution was obtained (10 mg/ml) and was used immediately.

Preparation of thymoquinone solution

Ten mg of thymoquinone was dissolved in 1 ml propylene glycol (PG) and was used within 1 h.

Animals and experimental design

The present research was carried out at King Fahd Research Center between June and November 2016, and the experimental protocol was approved by a unit of Biomedical Ethics Committee for Research (Certificate no. 68404/37/D; Dated: 27/01/2016) at Faculty of Medicine, King Abdul-Aziz University, Jeddah, KSA. A total of 48 male Wister rats weighing between 250 ± 25 g were used in this study. The rats were acclimatized for 1 week and housed in plastic cages (six rat each) in a temperature controlled (22–24 °C) room with a 12-h light/dark cycle and were allowed free access to standard rat food and water. All animals received good care complying with ethical standards. The study was carried out in two independent phases: phase I aimed to study the effect of TQ on bioavailability of CsA. Phase II was conducted to study the protective effect of TQ against CsA induced nephrotoxicity and hyperglycaemia. In all experiments the dose of TQ as well as CsA was 10 mg/kg, chosen on basis of previous study and previous work done in our lab for CsA.[10,11]

Experimental design for bioavailability study

Rats were randomly divided into four groups ($N = 6$). In each group, TQ was given daily for 7 days before starting CsA administration to insure sufficient time to demonstrate effect TQ on liver and GIT metabolizing enzymes. TQ (10 mg/kg) was administered orally and CsA was given either PO or IP at dose (10 mg/kg) in groups involving both TQ and CsA, CsA was given after 1 h of TQ administration. Group 1 received the vehicle alone (PG) for 1 week followed by CsA (PO) for 5 days with continuing of PG administration. Group 2 received TQ for 1 week followed by CsA (PO) for 5 days with continuing of TQ administration. Group 3 was given vehicle alone for 1 week followed by CsA (IP) for 5 days with continuing of PG administration. Group 4 received TQ for 1 week followed by CsA (IP) for 5 days with continuing of TQ administration. At day 12, blood samples were obtained from retro-orbital venous plexus from each rat using capillary tubes (Micro Hematocrit Capillaries, Mucaps) 1, 2, 3 and 5 h from CsA administration. Approximately 200 µl of whole blood was collected in ethylenediaminetetraacetic acid (EDTA) tube and stored at 4 °C for CsA analysis carried 147 within 7 days.

Cyclosporine A analysis

Cyclosporine A plasma level was determined using quantitative immunoassay that measured CsA in whole blood on the Dimension Vista® system using CSAE Flex® reagent cartridge provided from Siemens Healthcare Diagnostic Inc. (Henkestr., Erlangen, Germany). Blood samples were diluted before analysis with blood from untreated rats to obtain CsA concentrations within the range of the standard curve. Sampling, reagent delivery, mixing and processing are automatically performed by Dimension Vista® system. The analytical method was validated by using calibration control of CsA, coefficient of variation was less than 5% within concentration range of 5–200 ng/ml. Limited area under the CsA concentration curve (LAUC) was calculated for each rat to obtain relative bioavailability of CsA by using the linear trapezoidal method from time zero to time t, where t is the time for last concentration obtained. The AUC of CsA was estimated by using PK Solver 2.0.[12]

Experimental design for protective effect study

Rats were randomly divided into four different groups ($N = 6$). The dose of TQ, as well as CsA was 10 mg/kg as previously stated and administration was repeated daily for 28 days for all groups. CsA was given 1 h after oral administration of TQ solution. CsA was given IP in all groups whereas TQ was given PO. Group 1 was control receiving the vehicle (PG) alone, group 2 received TQ alone, group 3 comprised of both TQ + CsA (IP) given as described earlier and group 4 received CsA alone. The IP route of CsA administration was chosen based on bioavailability study which revealed no significant change in CsA (IP) bioavailability due to concomitant administration of TQ. All rats were weighted and observed for behavioural changes or mortality during the dosing and recovery period. At day 28, 2 ml of blood was collected in a plain tube, centrifuged at 3000*g* for 15 min and serum was separated and stored at −80 °C until used for kidney function analysis (cystatin C, and serum creatinine). For blood glucose analysis one drop of blood was taken from each rat and assay was performed by Bayer's CONTOUR® XT Meter according to the manufacturer's instructions.

All animals were sacrificed under ether anaesthesia, kidneys were dissected out and slices of fresh kidney tissues were cut and fixed in 10% buffered neutral formalin fixative for 24 h. Following fixation, the tissues were washed and processed through an ascending series of alcohol (70%, 90% and 100%), cleared in xylene and infiltrated with wax at 57 °C. Sections of 5-µm thickness were cut from the embedded tissues, stained by aqueous haematoxylin and alcoholic eosin and examined by Nikon microscopy at a magnification of ×1000.[13]

Renal function assessment

Assessment was done using commercially available diagnostic kits provided from Siemens Healthcare Diagnostics Inc.

Serum creatinine & Cystatin C analysis

Serum creatinine and Cystatin C levels were determined using URCA Flex® reagent and CYSC 186Flex® reagent cartridge respectively. 187 Measurement was done in a fully automated Dimension Vista® system.

Blood glucose assessment

Blood sample were taken as a drop after overnight fasting by using retro-orbital puncture. The blood sample was applied on the test strip and inserted on Contour XT meter which automatically generated the reading.

Statistical analysis

The obtained data were presented as the mean ± standard deviation (M ± SD). One way analysis of variance (ANOVA) followed by *post hoc* test was carried out and statistical comparisons among groups were performed using a statistical package program (SPSS, version 17.0). Pharmacokinetic data were analysed using an independent sample *t*-test. All *P* values are two-tailed, and $P < 0.05$ was considered as significant for all statistical analyses in this study.

Results

Effect of TQ on relative bioavailability of CsA

Table 1, Figures 1 and 2 summarize the effect of TQ on mean AUC (1–5 h) (ng h/ml) of CsA after its repeated administration (IP/oral) (10 mg/kg) for 5 days either alone or 1 h after oral administration of TQ solution (10 mg/kg). The mean AUC of CsA (PO) alone was 17 725 ± 6037 and AUC of CsA (PO) + TQ (PO) was 11 995 ± 2218. The mean % decrease in oral bioavailability of CsA was about 32% ($P > 0.05$). On the other hand, the mean AUC of CsA (IP) alone was 19 691 ± 3231 and AUC of CsA (IP) + TQ (PO) was 20 541 ± 8345. TQ produce unremarkable (4% increase) effect on AUC of CsA after its repeated IP administration.

Effect on serum creatinine level

As shown in Figure 3, the mean level of serum creatinine was significantly ($P < 0.05$) increased after 28 days of administration CsA (alone) compared with control, TQ, however, the concomitant administration of TQ with CsA slightly ($P > 0.05$) attenuated the increase in serum creatinine level.

Effect on Cystatin C level

As shown in Figure 4, the mean level of cystatin C was significantly ($P < 0.05$) increased after 28 days of administration of CsA (alone) compared with control, TQ and

Table 1 AUC (1–5 h) (ng h/ml) of cyclosporine A after repeated administration (PO or IP for 5 days) either alone or 1 h after thymoquinone (PO for 12 days) (both at dose of 10 mg/kg)

Groups	AUC (ng h/ml)	% Changes
CsA (PO)	17 725 ± 6037	32% Decrease
CsA (PO) + TQ	11 995 ± 2218	
CsA (IP)	19 691 ± 3231	4% Increase
CsA (IP) + TQ	20 541 ± 8345	

All data show insignificant difference ($P > 0.05$).

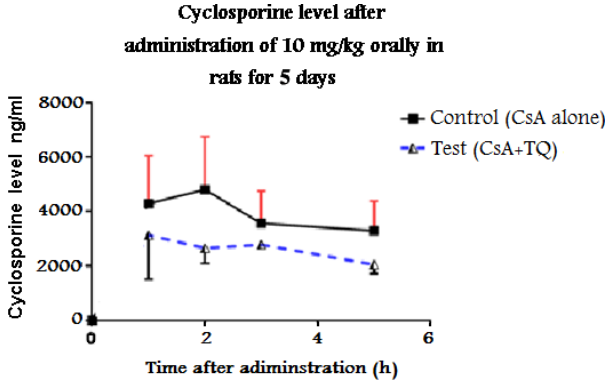

Figure 1 AUC of CsA (means ± SD) following its oral administration at a dose (10 mg/kg for 5 days) alone or 1 h after thyomquinone (10 mg/kg) in rats, (■cyclosporine A alone), (▲cyclosporine A + thyomquinone), Rats in cyclosporine A + thyomquinone group received thyomquinone alone (10 mg/kg, PO, for 7 days) before bio-availability experiment. [Colour figure can be viewed at wileyonlinelibrary.com]

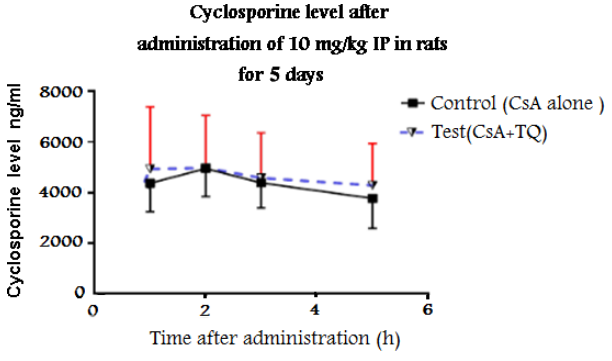

Figure 2 AUC of CsA (means ± SD) following its IP administration at a dose (10 mg/kg for 5 days) alone or 1 h after thyomquinone (10 mg/kg, orally) in rats, (■cyclosporine A alone), (▽cyclosporine A + thyomquinone), Rats in cyclosporine A + thyomquinone group received thyomquinone alone (10 mg/kg, orally, for 7 days) before bio-availability experiment. [Colour figure can be viewed at wileyonlinelibrary.com]

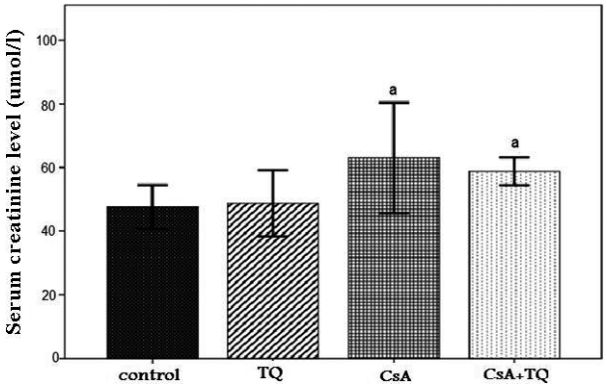

Figure 3 Mean of serum creatinine (μmol/l) in control, thyomquinone (10 mg/kg), cyclosporine A (10 mg/kg) and cyclosporine A (10 mg/kg) + thyomquinone (10 mg/kg) group (duration of treatment for 28 days). 'a' represents significance ($P < 0.05$) vs control and thyomquinone group.

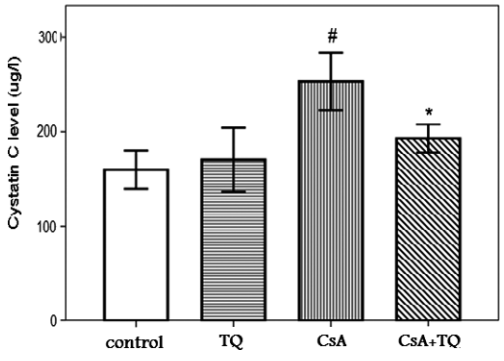

Figure 4 Effect of cyclosporine A (10 mg/kg), thyomquinone (10 mg/kg), cyclosporine A (10 mg/kg) + thyomquinone (10 mg/kg) and control group on mean of cystatin C (μg/l) (duration for 28 days). #Significant ($P < 0.05$) vs control, thyomquinone and cyclosporine A + thyomquinone group. *Significant ($P < 0.05$) vs cyclosporine A group.

CsA + TQ group. However, concomitant administration of TQ with CsA significantly ($P < 0.05$) attenuated the increase in cystatin C level.

Effect on blood glucose level

The level of blood glucose significantly decreased in TQ + CsA group as compared with CsA group alone. The results are summarized in Figure 5.

Histopathology of the kidney

The kidney obtained from control group (Figure A1–A2 in Appendix) showed similar features to those described in literature. The parenchyma consists of an outer cortex and inner medulla. The cortex showed numerous renal corpuscles (Bowman capsule + glomerular capillaries). Tubules were mainly proximal (PT) with narrow lumina and lined by pyramidal cells, having acidophilic cytoplasm and basal rounded nuclei. Distal tubules showed wider lumina, its cells showed less height compared with those of proximal tubules. Blood capillaries between tubules were compressed and non-congested. In group treated with CsA for 28 days (Figure B1–B2 in Appendix) kidney parenchyma showed mild to moderate changes in glomerular capillaries that looked shrunken and lobulated. Distal tubules were more affected compared with proximal. They appeared dilated due to degeneration of their lining cells. Their lumina showed hyaline casts mixed with degenerated desquamated cells and nuclei. Severely affected animals that died during

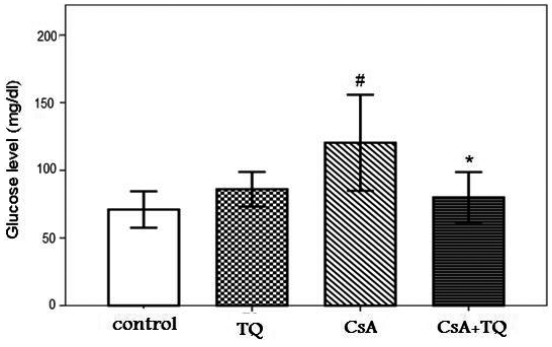

Figure 5 Level of glucose (mg/dl) in control, thyomquinone, cyclosporine A and cyclosporine A + thyomquinone group (both group at a dose 10 mg/kg for 28 days). *Significant ($P < 0.05$) vs control, thyomquinone and cyclosporine A + thyomquinone group. #Significant ($P < 0.05$) vs cyclosporine A group.

CsA treatment showed marked atrophy of both glomerular capillaries and renal tubules. In group treated with (CsA + TQ) (Figure C1–C2 in Appendix) potential protection against nephrotoxic histological changes induced by CsA were observed. There was potential preservation of renal corpuscle structure (Bowman capsules + glomeruli). Most distal tubules retained their normal structure, only few ones showed desquamated cells. Kidneys of animals receiving TQ alone (Figure D1–D2 in Appendix) looked histologically more healthy compared with control. Both renal corpuscle with its glomerular capillaries and renal tubules showed well-delineated integral structure. Tubular lining epithelium showed increased acidophilic staining. Peritubular capillaries also looked normal.

Discussion

Cyclosporine is an essential drug given to transplant patients, chronic administration of which induces potential harmful effects in the body. The most pronounced side effects of chronic CsA administration in patients are nephrotoxicity and hyperglycaemia.[2] Thus, rational use of a protective drug or herbal medicine could help in reducing these drug induced toxicity. Thymoquinone an active component of *N. sativa*, is well-known for its protective effects against renal injuries caused by various drugs.[14] Being an excellent antioxidant, it shows potential ameliorative effect on hyperglycaemic parameters as well.[15] Thus, the protective effect of TQ on various markers of hyperglycaemia in blood and histopathological changes of kidney were explored in this study.

For rational use of any supplemental herb, the study of its pharmacokinetic interactions with candidate drug is of foremost importance. Hence, in the first phase of this study, effect of TQ on bioavailability of CsA was investigated by administering CsA through oral as well as IP routes. It was demonstrated that an oral administration of TQ could cause a considerable decrease (about 32%, $P > 0.05$) in the bioavailability of the orally administered CsA. However, insignificant effect of TQ on bioavailability of CsA administered via IP route was observed which might be because the major site of CsA metabolism is the small intestines.[16] On other hand; TQ has a complex effect on metabolizing enzymes. Studies have demonstrated that it inhibits certain Phase I metabolizing enzymes CYP1A2, CYP3A4, but induces some of phase II pathways :glutathione-S-transferase (GST) and glutathione peroxidase (GPx).[17] In the present study, it was obvious that IP administration of CsA, avoided the first pass metabolism that has been induced by repeated administration of TQ. Similar pharmacokinetic interaction between CsA and *N. sativa* extract has been demonstrated where it significantly reduced bioavailability of oral CsA. The authors suggested that *N. sativa* extract induced intestinal P-glycoprotein and/or hepatic CYP3A activity.[18] However, in our study absence of significant interaction in case of IP CsA clearly indicated that the interaction is mainly mediated through inducing effect of TQ on intestinal first pass metabolism.

It was observed that a concomitant administration of TQ with CsA caused a significant reversal of high blood glucose levels. Many other studies have shown TQ to ameliorate the hyperglycaemic effects induced by some other drugs.[19,20] To the best of our knowledge, this is the first in vivo study which shows the anti-hyperglycaemic effect of pure TQ on CsA induced hyperglycaemia. The nephrotoxic effects induced by CsA, marked by serum creatinine level, were also slightly attenuated by coadministration of TQ. This finding is in line with previous studies showing an improvement in increased serum creatinine due to nephrotoxicity by TQ or *N. sativa* extracts.[21,22] Cystatin C is reported as a biomarker for prediction of early acute kidney injury (AKI).[23] Since a chronic administration of CsA is known to cause renal damage, our results also showed an increased level of Cystatin C in groups receiving CsA. In the present study, concomitant administration of TQ with CsA significantly attenuated the increase in cystatin C level. This is the first study of its kind which shows a direct effect of TQ on serum cystatin C levels thus pronouncing its protective effect on nephrotoxicity.

In the current study, histological assessments confirmed the nephrotoxicity after chronic administration of CsA marked by damage of both glomerular and tubular renal structures in rat kidney. Coadministration of TQ was found to prevent the major structural changes induced by CsA in both glomerular and tubular components. Also, administration of TQ alone was observed to show an enhanced health of kidneys when compared with control. This nephroprotective effect was attributed to the antioxidant

properties of TQ that counteract the pro-oxidative features of CsA underlying nephrotoxicity. Similar results have been previously obtained upon administration of TQ with vancomycin – where it reduced structural kidney damage.[24] Thus, TQ seemed to have two independent effects, 32% reduction of CsA oral bioavailability ($P > 0.05$) and attenuation of CsA induced nephrotoxicity and hyperglycaemia. However, these findings cannot be directly linked to those of humans, hence further comprehensive studies are highly recommended.

Conclusion

In conclusion, TQ proved to be an efficient protective agent against CsA induced nephrotoxicity and hyperglycaemia, not interfering with the bioavailability of IP administered CsA but only lowering its oral bioavailability. Thus considering the clinical relevance of these findings in human subjects with proper dosing, TQ could be developed as a protective drug where chronic administration of CsA is needed, especially in transplant patients.

Declaration

Acknowledgement

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Appendix

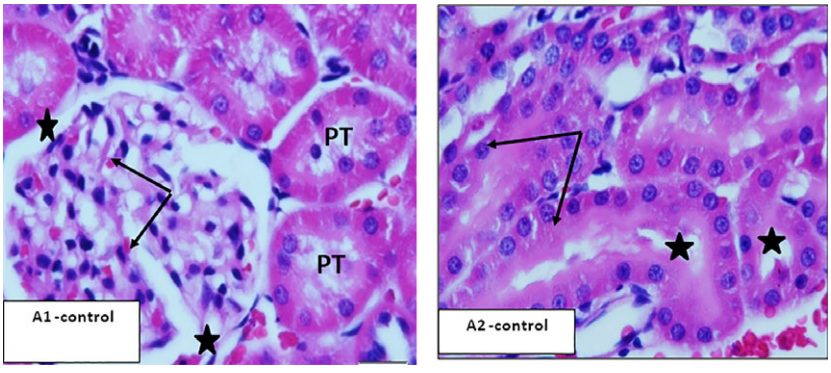

Figure A1–A2 Control showing: (A1) normal structure of renal corpuscle with intact normal glomerular capillaries (thin black arrows) separated from the outer layer of Bowman capsule by narrow space (black star). (A2) Renal tubules with its epithelium looked normal (black arrows). Their lumina are normal and free of any casts or cell debris (black stars) (H&E x1000). [Colour figure can be viewed at wileyonlinelibrary.com]

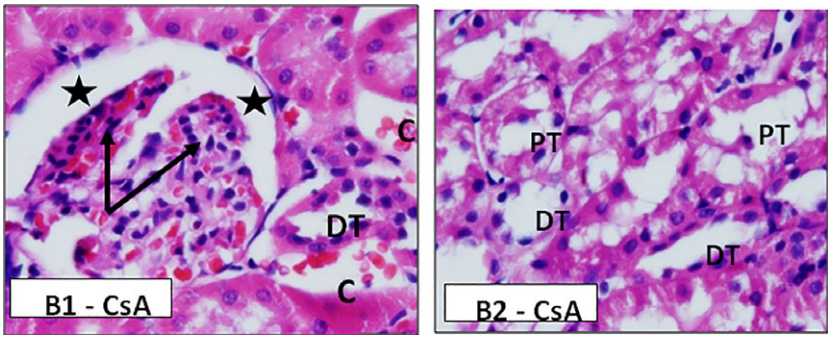

Figure B1–B2 Cyclosporine A showing (B1) features of nephrotoxic effect as sclerosis, shrinkage and lobulation of glomerular capillaries (thin black arrows) with an increase of Bowman space (black star), atrophy of distal tubule lining cells and capillary congestion (C). (B2) Some samples showed marked disorganization (D), atrophy and vacuolation of lining epithelium of both proximal and distal tubules (H&E x1000). [Colour figure can be viewed at wileyonlinelibrary.com]

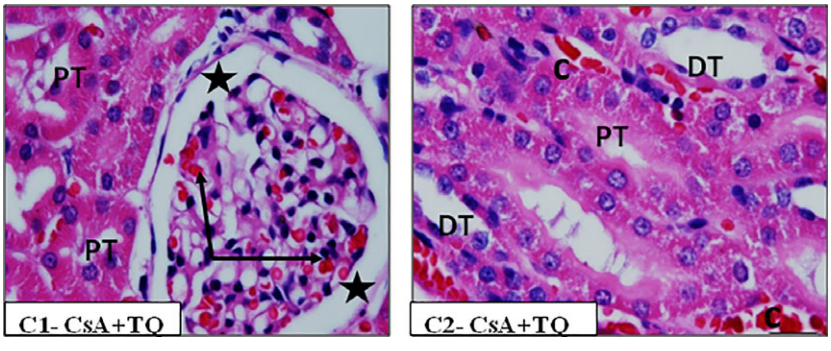

Figure C1–C2 Test group (cyclosporine A + thyomquinone) showing (C1) potential preservation of renal corpuscle structure, glomeruli capillaries looked normal slightly congested (black arrows), decrease widening of Bowman space (black stars). (C2) Renal tubules (PT) also looked normal with intact non-atrophied epithelial lining (H&E stain x1000). [Colour figure can be viewed at wileyonlinelibrary.com]

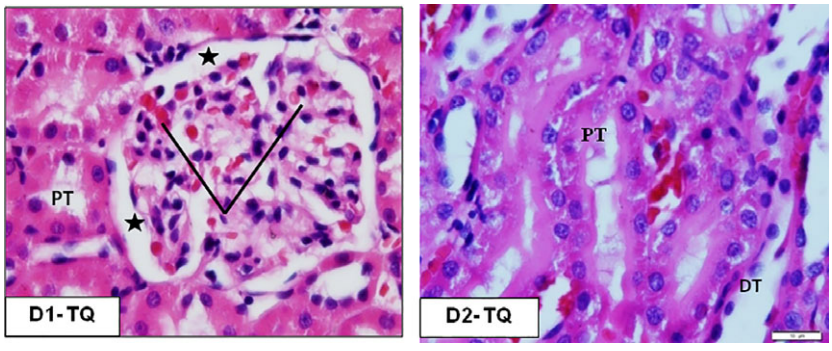

Figure D1–D2 Thyomquinone group showing (D1) renal corpuscle showed normal capsular space (stars) with more integrity and healthy appearance of renal glomeruli (black arrows) and tubules (PT). (D2) Both proximal and distal tubules looked normal (H&E x1000). [Colour figure can be viewed at wileyonlinelibrary.com]