Original Research Article

Interaction of cyclosporine with pomegranate juice and its potential nephroprotective effect in rats

Abstract

1. Chronic use of cyclosporine is associated with nephrotoxicity. Intestinal CYP450 and P-gp are major sources of cyclosporine absorption variability. Pomegranate juice is known to inhibit CYP450 in rodents.

This study aims to examine the effect of pomegranate juice on the bioavailability of cyclosporine and its role in preventing cyclosporine-induced nephrotoxicity in rats.

Male Wistar rats were randomized to receive 20 mg/kg oral or SC cyclosporine 1 hour after either pomegranate juice (2 mL) or distilled water for 5 days (n=6). Blood samples were taken to measure cyclosporine levels. In a separate study, rats were randomized to receive oral cyclosporine, pomegranate juice or both for 28 days.

Results demonstrate that pomegranate juice lead to a 54 % increase in oral bioavailability of cyclosporine. In addition, co-administration of pomegranate juice with oral cyclosporine attenuated major biochemical and structural changes in the kidneys after 28 days of cyclosporine use. This study provided evidence On the other hand, pomegranate juice was found to amoleriate prevented cyclosporine-induced nephrotoxicity in rat.. Future studies are warranted to determine the risk and benefit of this combination in patients on cyclosporine based immunosuppressant regimen .

Keywords: pharmacokinetics; pomegranate juice; cyclosporine; food-drug interaction; P-gp; Cyp3a; nephroprotective

Introduction

Cyclosporine is a calcineurin inhibitor immunosuppressant drug commonly used in the management of solid organ transplantation as well as several autoimmune diseases (Piedras et al., 2013). The use of cyclosporine has led to substantial progress in the field of immunosuppression and its efficacy and toxicity are related to its blood level. Moreover , it has a narrow therapeutic range , and variable pharmacokinetics making therapeutic drug monitoring a mandatory requirement to optimize its use in clinical practice . Cyclosporine A has variable gastrointestinal absorption, which is influenced by many factors. These include numerous food-drug interaction, drug-drug interactions and disease-drug interactions with cyclosporine were reported (Wadhwa et al., 1987). Another important source for pharmacokinetic variability is that cyclosporine is subjected to extensive hepatic and intestinal metabolism by cytochrome P450 3A (CYP3A4/5) (Kronbach et al., 1988). This makes cyclosporine pharmacokinetics affected by inhibitors and inducers of CYP3A metabolism (Benet, 2009). An increasing number of drugs and herbals have been labelled as having a clinically significant interaction with cyclosporine due to interactions with CYP450 enzymes (Michalets, 1998). Cyclosporine is also a substrate for the major drug efflux transporter; P- glycoprotein (P-gp) (Saeki et al., 1993, Lown et al., 1997). P-gp is located in numerous sites including the small intestine, and is a known source of serious drug-drug and drug-food interactions (Konig et al., 2013). Interaction of cyclosporine with intestinal P-gp is considered a major source of variability in the pharmacokinetics of cyclosporine. In this context 17% of the variability in oral cyclosporine pharmacokinetics is attributed to the amount of intestinal P-gp (Hebert, 1997). Maintaining tight control of cyclosporine levels within the target range is essential for

efficacy and minimizing adverse effects y (Kahan et al., 2000).

One of the most serious adverse effects associated with cyclosporine therapy is nephrotoxicity (Bennett, 1996). The difficulty is balancing the risk of organ rejection as a result of inadequate cyclosporine exposure to the risk of nephrotoxicity; which is a result of over exposure (Tedesco and Haragsim, 2012). Cyclosporine induced nephrotoxicity is either acute and reversible, or chronic and irreversible. Chronic nephrotoxicity is described as progressive and irreversible renal dysfunction associated with morphological changes in the kidney including tubulo-interstitial injury and glomerulosclerosis (Mihatsch et al., 1988, Myers et al., 1984). The cause of chronic nephrotoxicity following the use of calcineurin inhibitors; such as cyclosporine is thought to be caused by both direct toxic effects of the drug as well as hemodynamic changes.. Several reactive oxygen species have been reported to be involved in cyclosporine-induced nephrotoxicity (Wong et al., 2002, O'Connell et al., 2012, Perez de Hornedo et al., 2007, Naesens et al., 2009). In addition, antioxidants have been reported to attenuate cyclosporine induced nephrotoxicity (Damiano et al., 2015). Pomegranate juice is a known antioxidant (Gil et al., 2000, Singh et al., 2002), which has been reported to display nephroprotective properties against a number of drug induced kidney injury in rodent models (Abdel Moneim and El-Khadragy, 2013, Tugcu et al., 2008, Moneim et al., 2011). Pomegranate juice is increasingly being used worldwide due to its numerous reported health benefits (Basu and Penugonda, 2009, Aviram et al., 2004, Pantuck et al., 2006). We hypothesize that the consumption of pomegranate juice would protect against chronic cyclosporine-induced nephrotoxicity. In this study we first examine the effect of concomitant administration of pomegranate juice on the bioavailability of cyclosporine in rats and then the potential protective effect of pomegranate juice on cyclosporine induced nephrotoxicity.

Materials and Methods

Chemicals and Reagents

Cyclosporine (Neoral, Novartis pharmaceuticals, Australia) obtained as an oral solution (100 mg/ml) and injection (50 mg/ml).

Preparation of Pomegranate Juice

Fresh pomegranate fruit (Punica granatum) was obtained from a local supplier as a single batch. The juice was obtained by squeezing the arils of pomegranate fruit using a commercial blender. The undiluted juice was then filtered through a stainless steel fine mesh strainer. Pomegranate juice was stored in small-capped amber glass containers (5 mL each) at -20 °C and used within a month.

Animals

Male Wister rats (300gm ± 25gm) were housed in plastic cages at constant temperature (22± 1°C) with a 12-h light/dark cycle. Standard rat chow and water were provided ad libitum. The experimental protocol was approved by the National Committee of Bio and Med ethics (Reference # 342-15) – King Abdulaziz City for Science and Technology and conducted at King Fahd Medical Research Centre. Animals were used in the first study to examine the interaction between cyclosporine and pomegranate juice. In the 2nd study animals were used to evaluate the long-term potential nephroprotective effect of pomegranate juice when co-administered with cyclosporine. The total number of animals included in the study (52 rats) was based on priori analysis using "GPower version 3.1" program, in order to detect a 30% difference between the means of 2 independent groups, when the standard deviation was assumed to be 20%, and a significance level was given 0.05 with a power of 90% to detect differences.

Pharmacokinetic study

Animals were randomized in the morning to first receive either oral pomegranate juice (PJ) (2 mL) or distilled water (DW) via gavage tube. One hour later, animals received cyclosporine (20 mg/kg) either orally (PO) via gavage tube or subcutaneously (SC) (n=6). Administration was repeated for 5 consecutive days. Multiple blood samples were collected on the first and last day of the study following , 1, 2, 3 and 5 hours of cyclosporine administration. For analysis of cyclosporine level, 200 µl of blood samples were obtained from the retro-orbital venous plexus using capillary tubes (Micro Haematocrit Capillaries, Mucaps) and collected in EDTA coated tubes.

All samples were kept at 4 °C and analysed within 7 days. To examine the effect of pomegranate juice in preventing cyclosporine-induced nephrotoxicity, a second study was conducted. In this study rats were randomized to receive a once daily dose of oral cyclosporine (20 mg/kg) (n=7), oral pomegranate juice (2 mL) (n=9) or sterile distilled water orally (2 mL) as a control group (n=5). In addition, one group received both pomegranate juice and oral cyclosporine (13 mg/kg) (n=7). The lower dose of cyclosporine was utilized when the combination was used in order to circumvent the increase in cyclosporine bioavailability following pomegranate juice administration, which we have already observed, from the first study. The lower dose of cyclosporine was calculated in order to achieve the same AUC as the 20 mg/kg of cyclosporine, using the following formula:

$$\text{Adjusted cyclosporine dose} = \frac{\text{dose} \times \text{desired AUC}}{\text{obtained AUC}}$$

Where the desired AUC: is the one obtained from giving oral cyclosporine alone as 20 mg/kg. The obtained AUC: is the one obtained from co-administration of

oral cyclosporine (20 mg/kg) and pomegranate juice. Administration was repeated for 28 days to ensure sufficient time for histological changes. At the end of the experiment animals were euthanized and both kidneys were removed for histopathological assessment. For analysis of peak and trough cyclosporine levels, 200 µl of blood samples were collected in EDTA coated tubes following 3 and 24 hours of cyclosporine administration. In addition, 1 mL of blood was obtained, centrifuged at 3000 rpm for 15 min to obtain the serum. Analysis of serum creatinine and blood urea nitrogen (BUN) were determined using Dimension Vista 1500 Intelligent Lab Systems (Siemens Healthcare, Erlangen, Germany).

Histopathology Assessment

At the end of the 28 day study, rat kidneys were extracted from anesthetized rats cut into fine slices (≈ 3 mm) and fixed for 48 hours in 10% neutral buffered formalin solution for further paraffin embedding ded then 5 micron thick sections were stained with haematoxylin/eosin stain; adopting standard histological techniques (Jensen, 2008). Briefly, fixed tissues were dehydrated through a series of graded ethanol bathes using 70 and 95% ethanol solutions. Xylene was used for clearing the fixed tissues then infiltrated with paraffin wax (melted at 58-60 °C) then embedded into wax blocks. Slices of 5 µm were made, stained by aqueous haematoxylin and eosin and examined under the microscope (Nikon microscope x400 magnification). Photographing was done to compare histological structure of both control and experimental groups using light microscopy (Nikon Eclipse TE2000-U, NIKON,)

Japan)

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Cyclosporine Analysis

Cyclosporine blood level was determined by an automated immunoassay procedure using Dimension Vista 1500 Intelligent Lab Systems, which utilizes CSAE Flex® reagent cartridge (Siemens healthcare diagnostic Inc., Erlangen, Germany). Whenever necessary rat blood samples were appropriately diluted with untreated-rat whole blood. Accuracy of analysis was confirmed using three levels of calibration control and the coefficient of variation was less than 5 %.

Cyclosporine area under the concentration time curve from 0 to 5 hours (AUC0-5) was estimated using PKSolver add-in program for Microsoft Excel by Visual Basic for Application (VBA). The program utilizes the linear trapezoidal method to estimate AUC as a measure of relative cyclosporine bioavailability.

Statistical Analysis

Data from the pharmacokinetic study is presented as means ± standard deviation (SD). Statistical analysis was performed using PRISM 6 software. In the first study, a two-tailed student t-test was used to determine the effect of pomegranate juice administration on the bioavailability of cyclosporine. In the second study, a one-way ANOVA followed by Tukey's multiple comparison test was used to determine the effect of cyclosporine administration in the control group and the group that received both cyclosporine and pomegranate juice.

Results

Effect of pomegranate juice on the relative bioavailability of cyclosporine

Repeated administration of pomegranate juice and PO cyclosporine for 5 consecutive days lead to a significant increase in the mean AUC1-5 of cyclosporine 30702 ± 4249 ng.hr/mL compared to the control group 19191 ± 3741 ng.hr/mL (Table I). The mean percent increase in relative bioavailability was about 38% (Figure 1). On the other hand, repeated administration of pomegranate juice with SC cyclosporine did not lead to significant changes in the AUC of cyclosporine (Figure 2) (Table I). Single exposure (day 1) to pomegranate juice did not lead to significant changes in the AUC_{1-5} of cyclosporine regardless of the method of cyclosporine administration (Table I).

Effect of pomegranate juice on the peak and trough concentrations of cyclosporine

Peak and trough concentrations of cyclosporine 28 days following administration of PO cyclosporine 20 mg/kg and co-administration of cyclosporine 13 mg/kg with pomegranate juice were not significantly different (Table 2). These results confirmed that the two doses of oral cyclosporine (with and without pomegranate) gave comparable overall drug exposure ; to fairly allow comparing nephroprotective potential of pomegranate (independent of its effect on bioavailability).

Protection of pomegranate juice against cyclosporine induced nephrotoxicity

Long-term PO administration of cyclosporine (20 mg/kg) for 28 days resulted in a significant increase in serum creatinine and BUN compared with the control group (Table 3). In addition, cyclosporine resulted in major histopathological changes in the

renal cortex structures consistent with nephrotoxicity. This included glomerular lobulation, dilated distal tubules, the presence of intraluminal casts and desquamated cells (Figure 3c). Administration of pomegranate juice along with PO cyclosporine (13 mg/kg) was associated with significantly lower BUN level compared with cyclosporine alone, along with a trend towards lower serum creatinine level. In addition, the combination was only associated with few desquamated degenerated kidney cells and slight dilatation of peri-tubular capillaries (Figure 3d). On the other hand classic features of normal kidney tissues (glomeruli and tubules) were present when either pomegranate juice alone or distilled water were administered to control rats (Figure 3a-b).

Discussion

Chronic use of cyclosporine is an integral part of immunosuppressive therapy. However, it is susceptible to numerous interactions which may lead to significant clinical consequence (Fugh-Berman and Ernst, 2001). Consumption of pomegranate juice is popular around the around, due to the number of documented health benefits it conveys (Aviram et al., 2004, Sumner et al., 2005, Basu and Penugonda, 2009, Pantuck et al., 2006, Esmaillzadeh et al., 2004).

In the present study, repeated daily administration of oral pomegranate juice lead to a significant increase in the oral bioavailability of cyclosporine . Similar pharmacokinetic interactions with pomegranate juice were documented with a number of other drugs including carbamazepine (Hidaka et al., 2005), nitrendipine (Voruganti et al., 2012) and buspirone (Shravan Kumar et al., 2011). In these studies, administration of pomegranate juice in animal models lead to significant increase in the peak plasma concentrations and bioavailability of these drugs. While the exact mechanism behind

such an interaction has not been examined in our current study, many in vitro and in vivo studies suggest that pomegranate juice inhibits CYP450 enzymes, particularly CYP3A and CYP2C9 (Srinivas, 2013, Hidaka et al., 2005, Nagata et al., 2007, Faria et al., 2007, Kim et al., 2006, Summers, 2006). This may explains the increase in cyclosporine bioavailability following pomegranate juice administration in our study. Pomegranate juice has also been reported to inhibit the efflux transporter P-gp (Voruganti et al., 2012) (Voruganti et al., 2012); leading to increased intestinal permeability of drugs and the fraction of drug absorbed. Moreover, it appears that inhibition of CYP3A4 and P-gp by pomegranate juice is more predominant in the intestine; more so than in the liver (Hidaka et al., 2005, Voruganti et al., 2012, Won et al., 2012, Won et al., 2010). Taken together, it is likely that the mechanism underlying the increase in cyclosporine bioavailability in our study following administration of pomegranate juice is probably due to inhibition CYP3A4/5 iso-enzymes and P-gp in the intestine. It also explains why the increase in cyclosporine bioavailability was only demonstrated following repeated oral but not SC cyclosporine administration, which likely by-passed the first pass effect in the intestine. In patients, significant interaction has been reported between a known inhibitor of CYP3A4 and P-gp; i.e grapefruit juice, and cyclosporine (Kiani and Imam, 2007, Hollander et al., 1995, Yee et al., 1995, Chan et al., 1998, Romiti et al., 2004). Accordingly, drug labelling recommendations advise patients to avoid the use of grapefruit juice with cyclosporine (Huang and Lesko, 2004).

Repeated administration of pomegranate juice in humans; with some known CYP3A substrates such as simvastatin (Park et al., 2016) and midazolam (Misaka et al., 2011, Farkas et al., 2007), as well flurbiprofen (Hanley et al., 2012); which is a substrate for CYP2C9, did not demonstrate inhibition of CYP450 isoforms, nor did it result in

pharmacokinetic alterations. Regarding the interaction of pomegranate juice with midazolam, Farkas et al suggested that the dissimilarity between the inhibition of CYP3A in rats and the lack of such an inhibition in humans could be a result of species differences in the metabolism and pharmacokinetics of midazolam (Farkas et al., 2007).

. We also demonstrate that the effect of pomegranate juice on cyclosporine bioavailability appears only after repeated but not after single administration. This suggests a dose-dependent inhibition of Cyp3a by pomegranate juice as previously reported (Hidaka et al., 2005).

In the current study, we provide evidence of potential nephroprotective effect of pomegranate juice against cyclosporine-induced nephrotoxicity. Histological assessments and biochemical changes confirmed the nephrotoxic effect of cyclosporine in rats following repeated daily exposure to PO cyclosporine for 28 days. This was demonstrated by the significant damage detected in the vascular and tubular renal structure, consistent with chronic cyclosporine-induced nephrotoxicity (Naesens et al., 2009). These structural changes; dominated by sclerosis are suggested to take place in a later phase of cyclosporine-induced nephrotoxicity attributed to the pro-oxidative features of cyclosporine (Sereno et al., 2014). Other features of cyclosporine-induced nephrotoxicity were confirmed including an elevation in serum creatinine and BUN. The co-administration of pomegranate juice with cyclosporine prevented major structural changes in both glomerular and tubular kidney components of rats treated with cyclosporine. Moreover, biochemical changes consistent with nephrotoxicity were also ameliorated (or reduced) . This nephroprotective effect is attributed to antioxidant properties of pomegranate juice that counteracted the pro-oxidative features of cyclosporine. Ellagic acid; which is a phenolic component present in pomegranate fruit (Wang et al., 2004) has been shown to ameliorate kidney, heart and liver damage

produced by cyclosporine in rats (Yuce et al., 2008). In addition, studies in animal models documented protective effect of pomegranate against a number of drug induced nephrotoxicity including gentamicin (Cekmen et al., 2013) and cisplatin (Bakir et al., 2015).

Conclusion

In conclusion, our study demonstrates that the oral bioavailability of cyclosporine is markedly increased after chronic administration of pomegranate juice. On the other hand, there is a protective effect of pomegranate juice against cyclosporine induced kidney injury in rats following repeated administration. Future studies are warranted to determine the risk and benefit of this combination in human

Data Availability Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Table 1. Mean cyclosporine AUC following single or repeated exposure to pomegranate juice (PJ) expressed as the means ± S.D.

Groups	**AUC_{1-5} (ng.hr/ml) 1st day (single)**	**AUC_{1-5} (ng.hr/ml) 5th day (repeated)**
Cyclosporine (PO) + DW	12,479 ± 2595	19,191 ± 4489
Cyclosporine (PO) + PJ	11,354 ± 1271	30,702 ± 5099**
Cyclosporine (SC) + DW	12,745 ± 1425	43,640 ± 8029
Cyclosporine (SC) + PJ	15,744 ± 8777	34,219 ± 9568

Table 2. Peak (Cmax) and trough concentrations (Cmin) of cyclosporine in rats following 28 days of administration expressed as the means ± S.D

Group	**Cmax (ng/ml)**	**Cmin (ng/ml)**

DW + Cyclosporine (PO) (20 mg/kg)	5190 ± 2908	2608.7 ± 2317
Pomegranate juice + Cyclosporine (PO) (13 mg/kg)	5700 ± 3300	2267 ± 1476

Table 3. Biochemical changes in rats following 28 days of administration presented as mean ± SD

	Control	**Pomegranate Juice (PJ)**	**Cyclosporine (Cs)**	**PJ + Cs**
BUN (mmol/L)	6.6 ± 0.9	6.6 ± 1.1	14.6 ± 6.4**	7.9 ± 1.8 ##
Creatinine (μmol/L)	40.6 ± 3.5	51.5 ± 4.7	63.4 ± 7.3**	56.3 ± 13.4

* Significant from control group, # Significant from cyclosporine (Cs) group.

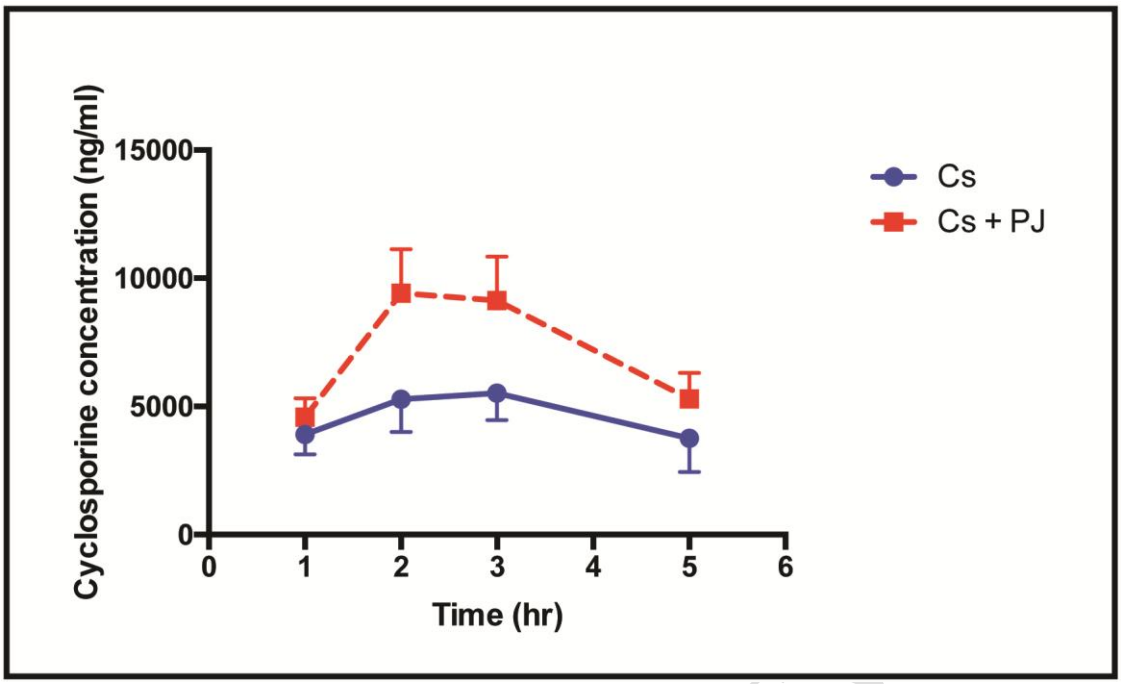

Figure 1. Effect of repeated pomegranate juice administration on the AUC of oral cyclosporine. Results show concentration time profile of cyclosporine on day 5 following oral administration of cyclosporine (Cs) with pomegranate juice (PJ) or distilled water for 5 days. Values are presented as mean±SD (n = 6 rats).

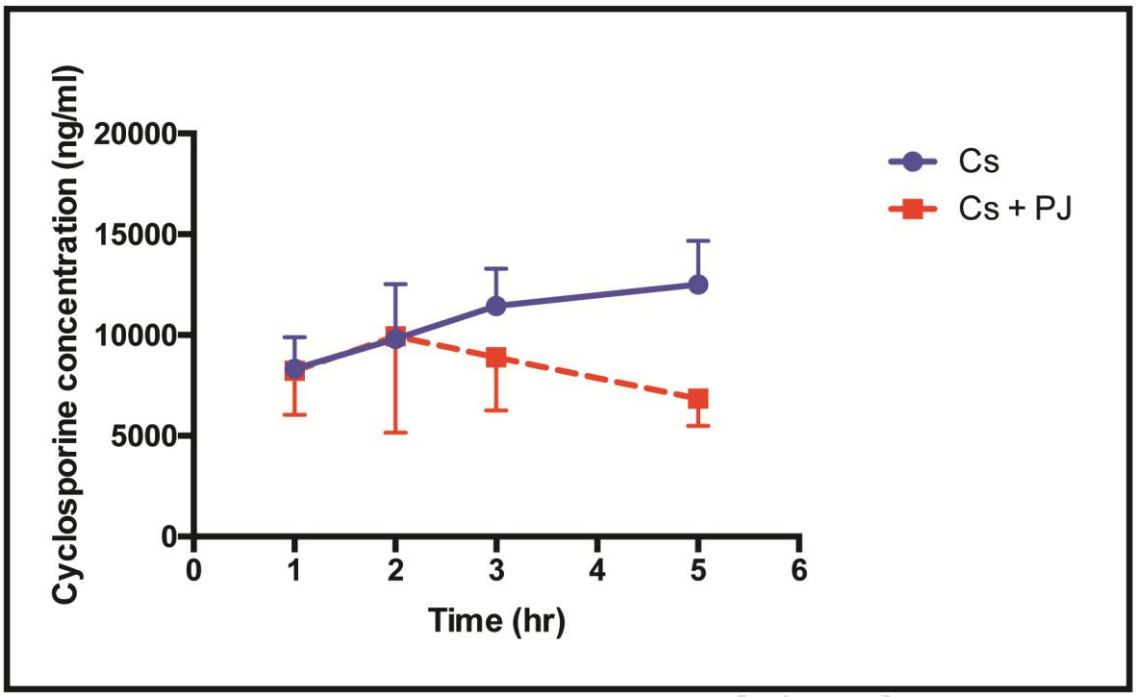

Figure 2. Effect of repeated pomegranate juice administration on the AUC of SC cyclosporine. Results show concentration time profile of cyclosporine on day 5 following SC administration of cyclosporine (Cs) with pomegranate juice (PJ) or distilled water for 5 days. Values are presented as mean±SD (n = 6 rats).

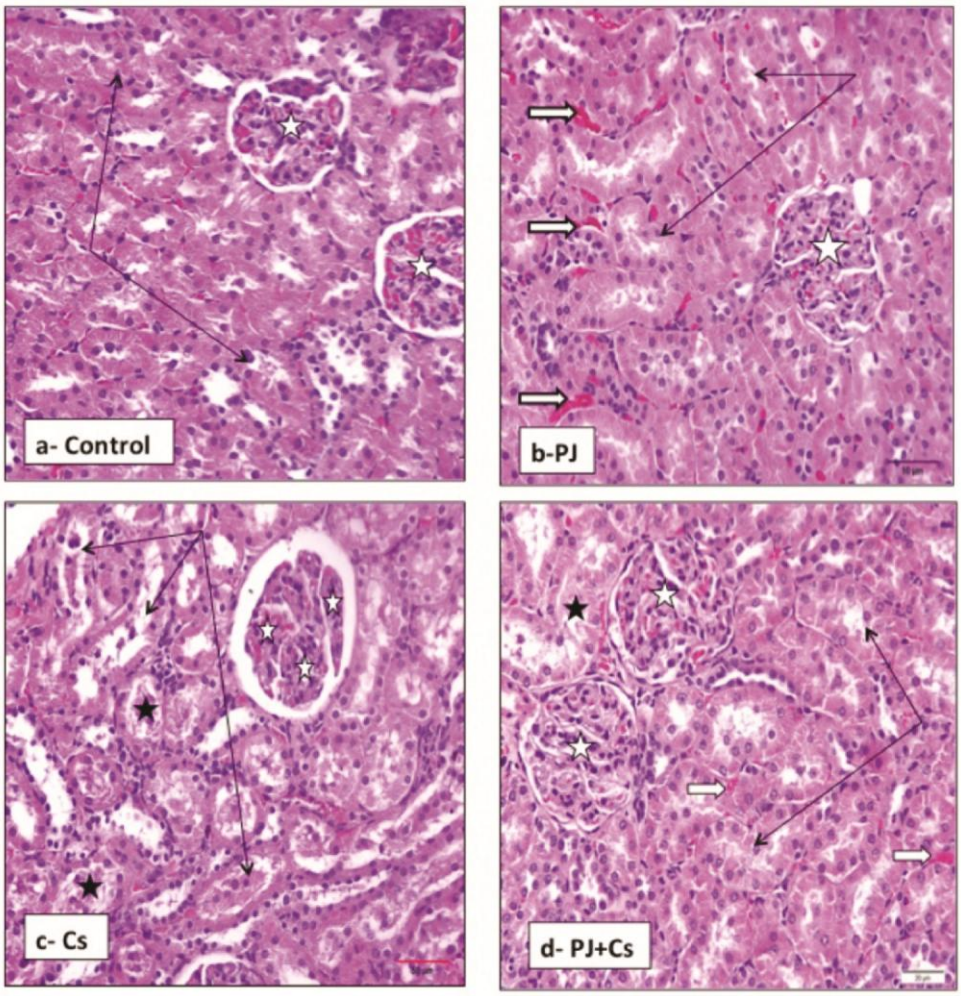

Figure 3. Histological examination of rat kidney cortex following long-term administration of SC cyclosporine and pomegranate juice. H&E stained sections from rat kidney cortex (magnification x400) showing (a) Control group showing normal structure of renal glomeruli (white stars) and tubules (thin black arrows). (b) Pomegranate (PJ) group showing normal renal glomeruli (white stars) and tubules (arrows). Peri-tubular capillaries looked slightly dilated (white arrows). (c) Cyclosporine group showing slight lobulation of renal glomeruli (white stars) and dilated distal tubules (black thin arrows) with intraluminal casts and desquamated cells (black stars). (d) PJ+ Cyclosporine group showing normal renal glomeruli (white stars) and tubules (black arrows). Few tubules showed desquamated degenerated cells (black star). Peri-tubular capillaries showed slight dilation (white arrows).