Original Research Article

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

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Abstract

Objectives To study the effect of concomitant administering of pomegranate juice orally (PJ) on bioavailability of cyclosporine A (CsA) . and independently its potential nephroprotective effect on CsA induced nephrotoxicity

. **Methods**; A- Pharmacokinetic (PK) , Wister rats were divided into groups (each group 6 rats)::. I-: CsA PO + Vehicle; , II- CsA IP + Vehicle , III- CsA PO + PJ , IV- CsA IP + PJ . CsA dose was 20 mg/kg for 5 days the vehicle or PJ (2 ml) was given 1h before drug administration . Blood samples were taken at the 5th day at specified times and CsA level was determined by immune assays.

B- Nephroprotection study (separate study to administer bioequivalent CsA Po dose, in view of PK)., I- (CSA 13 mg PO + 2ml PJ .II- CSA 20 mg P0 + 2 ml vehicle (for 28 day) . The design also include two control groups (vehicle alone or PJ alone). Blood samples for biochemical investigations and kidney samples for histopathology were taken at the 28th day.

Results PJ juice enhanced the bioavailability of oral CsA by about 50 % (P > 0.05). but CsA (IP) was not affected. Independently, the marked kidney damage induced by CSA was reversed by concomitant administration of PJ as well as the increase in serum creatinine.

Conclusions Repeated administration of pomegranate juice enhance CsA oral bioavailability which likely due to inhibition of intestinal enzymes and transport pump . Independently it caused significant attenuation of CsA induced renal toxicity

Keywords: pharmacokinetics; pomegranate juice; CsA; food-drug interaction; P-glycoprotein efflux pump (P-gp); CYP3A4; nephroprotective

Introduction

Cyclosporine A (CsA) is a calcineurin inhibitor immunosuppressant drug commonly used in the management of solid organ transplantation as well as several autoimmune diseases [1].., It has a narrow therapeutic range , and variable gastrointestinal absorption, which is influenced by many factors. such as numerous food-drug interaction, drug-drug interactions and disease-drug interactions with CsA were reported [2]. Another important source for pharmacokinetic variability is that CsA is subjected to extensive hepatic and intestinal metabolism by cytochrome P450 3A (CYP3A4/5) [3]. This makes CsA pharmacokinetics affected by inhibitors and inducers of CYP3A metabolism [4]. An increasing number of drugs and herbals have been labelled as having a clinically significant interaction with CsA due to interactions with CYP450 enzymes [5]. CsA is also a substrate for the major drug efflux transporter; P-glycoprotein (P-gp) which is a known important protein of the membrane that pumps many foreign substances out of cells. [6, 7]. P-gp is located in numerous sites including the small intestine, and is a known source of serious drug-drug and drug-food interactions [8]. Interaction of CsA with intestinal P-gp is considered a major source of variability in the pharmacokinetics of CsA. In this context 17% of the variability in oral CsA pharmacokinetics is attributed to the amount of intestinal P-gp [9]. Maintaining tight control of CsA levels within the target range is essential for efficacy and minimizing adverse effects y [10].

One of the most serious adverse effects associated with CsA therapy is nephrotoxicity [11]. The difficulty is balancing the risk of organ rejection as a result of inadequate CsA exposure to the risk of nephrotoxicity; which is a result of over exposure [12]. CsA induced nephrotoxicity is either acute and reversible, or chronic and irreversible. Chronic nephrotoxicity is a progressive and irreversible renal dysfunction

associated with morphological changes in the kidney including tubulo-interstitial injury and glomerulosclerosis [13, 14]. The cause of chronic nephrotoxicity following the use of calcineurin inhibitors; such as CsA 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 CsA-induced nephrotoxicity [15-18]. In addition, antioxidants have been reported to attenuate CsA induced nephrotoxicity [19]. Pomegranate juice is a known antioxidant [20, 21], which has been reported to display nephroprotective properties against a number of drug induced kidney injury in rodent models [22-24]. Pomegranate juice is increasingly being used worldwide due to its numerous reported health benefits [25-27]. We hypothesize that the consumption of pomegranate juice would protect against chronic CsA-induced nephrotoxicity. In this study we first examine the effect of concomitant administration of pomegranate juice on the bioavailability of CsA in rats and then the potential protective effect of pomegranate juice on CsA induced nephrotoxicity.

Materials and Methods

Chemicals and Reagents

Cyclosporine A (CsA) (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 unit of biomedical ethics research committee, faculty of medicine, king Abdul-Aziz university (No 14854 / 1437 H) and conducted at King Fahd Medical Research Centre.

Pharmacokinetic study

CsA), Pomegranate Juice (PJ), Oral gavage (PO), Intraperitoneal injection (IP) are the abbreviation used in the following design .

Wister rats were randomly divided into four groups (each 6) as follows :

I-: CSA (PO) + Vehicle	II- CSA (IP) + Vehicle
III- CSA (PO) + 2 ml PJ,	IV- CSA (IP) + 2ml PJ

CsA dose was 20 mg/kg, given once daily (in the morning) for 5 days , the vehicle or PJ was given 1h before drug administration

Sampling for drug analysis: 200 µl of blood samples was obtained from the retro-orbital venous plexus using capillary tubes (Micro Haematocrit Capillaries, Mucaps) and collected in EDTA coated tubes. The samples were taken on the 1st (single dose) and 5th day (multiple dose assuming steady state) of the study at specified time (limited area under curve approach) , 1, 2, 3 and 5 hours of post dose (CsA administration. ,:

Management of samples: All samples were kept at 4 °C and analysed within 7 days.

II- Potential Nephroprotection Study

Another group of rats, same as previously mentioned were randomized into groups

V – CsA (20 mg/kg) PO + vehicle (PO)	VI CsA (13 mg/kg) PO + 2 ml PJ (PO)

. The lower dose of CsA (13 mg/kg) +PJ was used in order to achieve the same AUC (i.e. relative bioavailability) as the 20 mg/kg of CsA with vehicle , (based on results of Pharmacokinetic study) using the following formula:

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

Administration was repeated for 28 days to ensure sufficient time for nephrotoxicity

Sampling for investigations: 1- Histopathology: At the end of the experiment animals were euthanized and both kidneys were removed for histopathological assessment. 2- peak and trough CsA levels, 200 µl of blood samples were collected as described under pharmacokinetic study , 3 hr post last dose (peak) and 24 hours post last dose (trough) . 3-Biochemistry, 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 then 5 micron thick sections were stained with haematoxylin/eosin stain; adopting standard histological techniques [28]. 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 Eclipse TE2000-U, NIKON, Japan).

Determination of CsA level

CsA 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 %.

CsA 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 CsA bioavailability.

Statistical Analysis

Data are presented as means ± standard deviation (SD). Statistical Package for Social Sciences (SPSS) software, version 22 was used for data analysis.In pharmacokinetic study, a two-tailed student t-test was used to compare the means of CsA levels. . In nephroprotective study, a one-way ANOVA followed by Tukey's multiple comparison test was used to compare mean biochemical values , A P value of < 0.05 was considered significant

Results

Effect of pomegranate juice on the relative bioavailability of CsA

Repeated administration of pomegranate juice and PO CsA for 5 consecutive days lead to a significant increase in the mean AUC1-5 of CsA 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 IP CsA did not lead to significant changes in the AUC of CsA (Figure 2) (Table I). Single exposure (day 1) to pomegranate juice did not lead to significant changes in the AUC_{1-5} of CsA regardless of the method of CsA administration (Table I).

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

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

Table 1. Mean CsA 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)**
CsA (PO) + DW	12,479 ± 2595	19,191 ± 4489
CsA (PO) + PJ	11,354 ± 1271	30,702 ± 5099**
CsA (IP) + DW	12,745 ± 1425	43,640 ± 8029
CsA (IP) + PJ	15,744 ± 8777	34,219 ± 9568

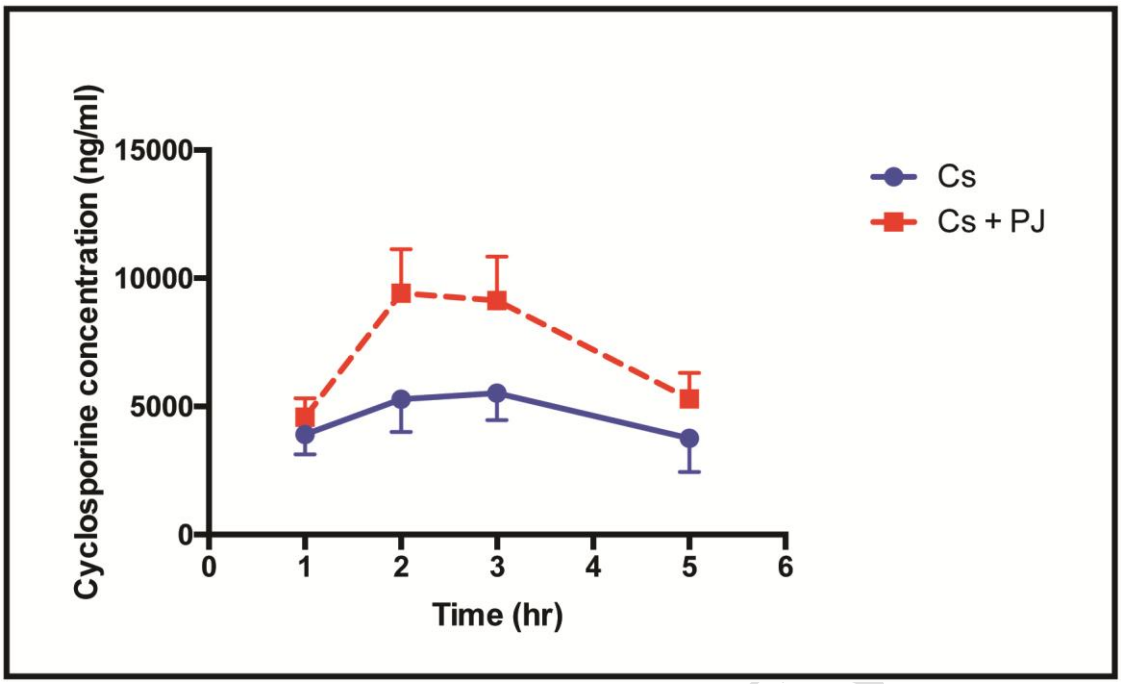

Fig 1. Effect of repeated pomegranate juice administration on the AUC of oral CsA. Results show concentration time profile of CsA on day 5 following oral administration of CsA (Cs) with pomegranate juice (PJ) or distilled water for 5 days. Values are presented as mean±SD (n = 6 rats

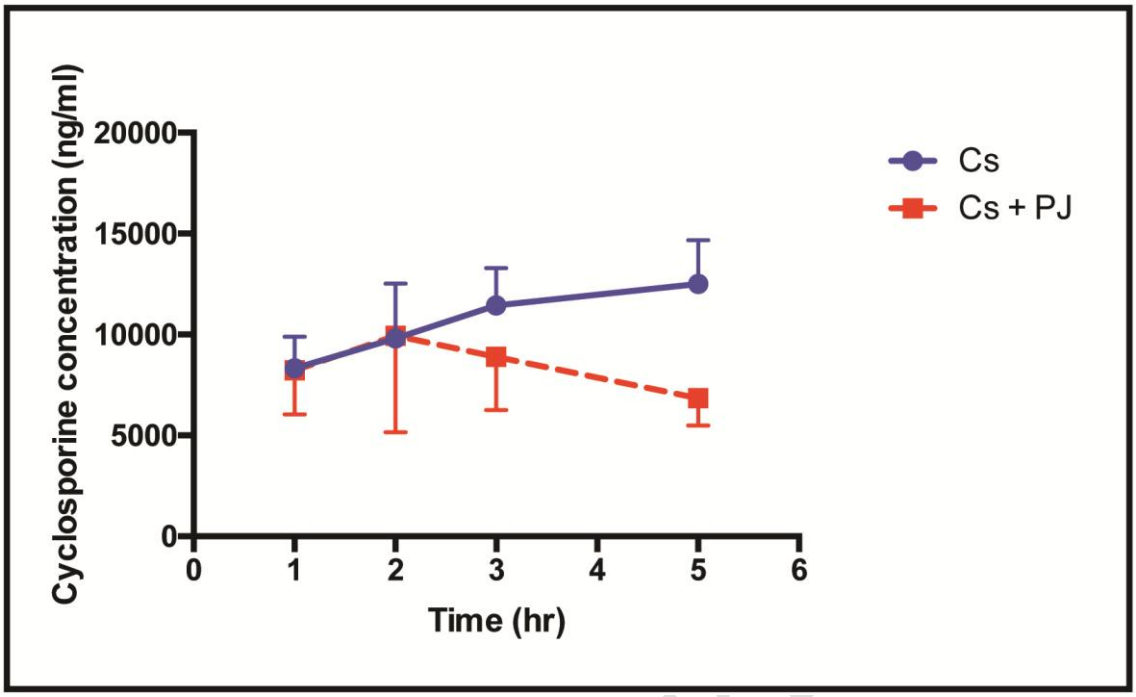

Figure 2. Effect of repeated pomegranate juice administration on the AUC of IP CsA. Results show concentration time profile of CsA on day 5 following IP administration of CsA (Cs) with pomegranate juice (PJ) or distilled water for 5 days. Values are presented as mean±SD (n = 6 rats).

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

Group	**Cmax (ng/ml)**	**Cmin (ng/ml)**
DW + CsA (PO) (20 mg/kg)	5190 ± 2908	2608.7 ± 2317
PJ + CsA (PO) (13 mg/kg)	5700 ± 3300	2267 ± 1476

Protection of pomegranate juice against CsA induced nephrotoxicity

Long-term PO administration of CsA (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, CsA 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 CsA (13 mg/kg) was associated with significantly lower BUN level compared with CsA 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).

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

	Control	**(PJ)**	**CsA**	**PJ + CsA**
BUN (mmol/L)	6.6 ± 0.9	6.6 ± 1.1	14.6 ± 6.4**	7.9 ± 1.8 ##
Serum 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 CsA group.

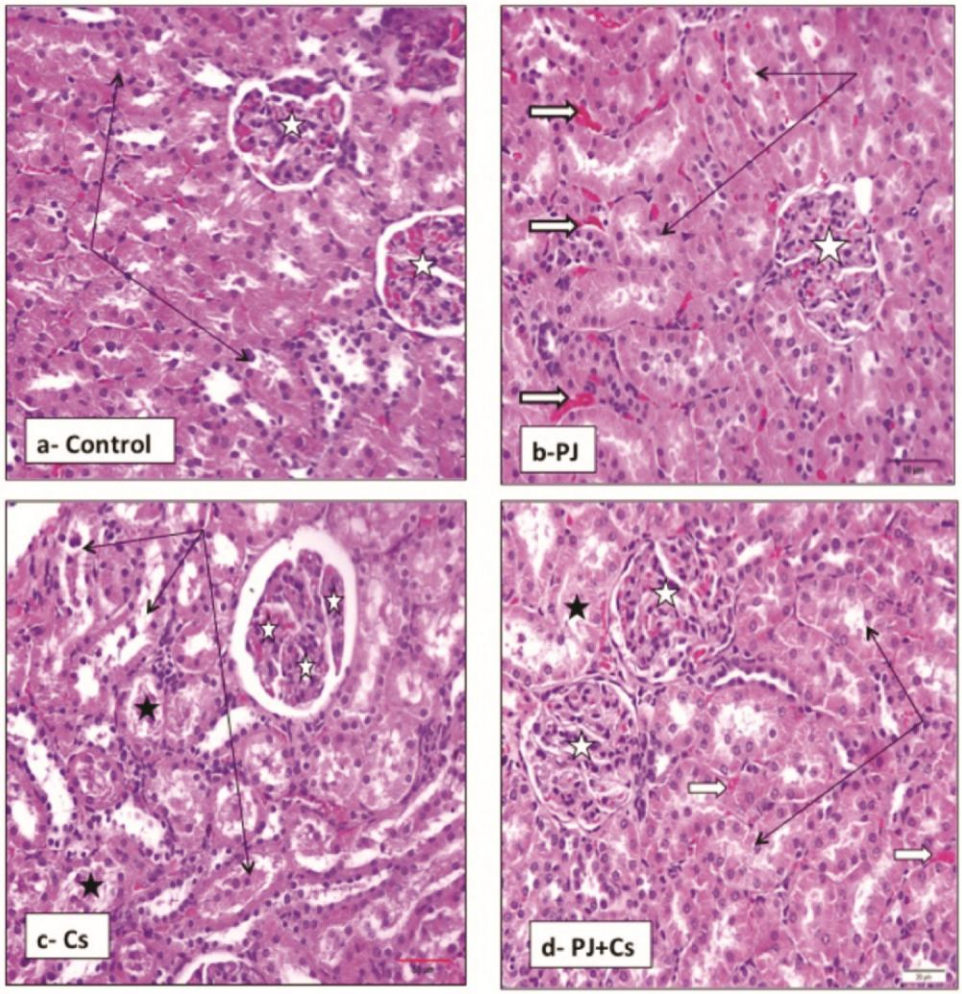

Figure 3. Histological examination of rat kidney cortex following long-term administration of CsA and pomegranate juice. H&E stained sections from rat kidney cortex (magnification x400) showing (a) Control group showing normal structure of renal glomeruli (white stars) and tubules (thin black arrows). (b) Pomegranate (PJ) group showing normal renal glomeruli (white stars) and tubules (arrows). Peri-tubular capillaries looked slightly dilated (white arrows). (c) CsA 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+ CsA 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).

Discussion

Chronic use of CsA is an integral part of immunosuppressive therapy. However, it is susceptible to numerous interactions which may lead to significant clinical consequence [29]. Consumption of pomegranate juice is popular around the around, due to the number of documented health benefits it conveys [25-27, 30, 31].

In the present study, repeated daily administration of oral pomegranate juice lead to a significant increase in the oral bioavailability of CsA. Similar pharmacokinetic interactions with pomegranate juice were documented with a number of other drugs including carbamazepine [32], nitrendipine [33] and buspirone [34]. 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 [32, 35-39]. This may explains the increase in CsA bioavailability following pomegranate juice administration in our study. Pomegranate juice has also been reported to inhibit the efflux transporter P-gp [33] [33]; 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 [32, 33, 40, 41]. Taken together, it is likely that the mechanism underlying the increase in CsA 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 CsA bioavailability was only demonstrated following repeated oral but not IP CsA 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 CsA [42-46]. Accordingly, drug labelling recommendations advise patients to avoid the use of grapefruit juice with CsA [47].

Repeated administration of pomegranate juice in humans; with some known CYP3A substrates such as simvastatin [48] and midazolam [49, 50], as well flurbiprofen [51]; 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 [50].

. We also demonstrate that the effect of pomegranate juice on CsA bioavailability appears only after repeated but not after single administration. This suggests a dose-dependent inhibition of Cyp3a by pomegranate juice as previously reported [32].

In the current study, we provide evidence of potential nephroprotective effect of pomegranate juice against CsA-induced nephrotoxicity. Histological assessments and biochemical changes confirmed the nephrotoxic effect of CsA in rats following repeated daily exposure to PO CsA for 28 days. This was demonstrated by the significant damage detected in the vascular and tubular renal structure, consistent with chronic CsA-induced nephrotoxicity [18]. These structural changes; dominated by sclerosis are suggested to take place in a later phase of CsA-induced nephrotoxicity attributed to the pro-oxidative features of CsA [52]. Other features of CsA-induced nephrotoxicity were confirmed including an elevation in serum creatinine and BUN. The co-administration of pomegranate juice with CsA prevented major structural changes in both glomerular and tubular kidney components of rats treated with CsA. 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 CsA. Ellagic acid; which is a phenolic component present in pomegranate fruit [53] has been shown to ameliorate kidney, heart and liver damage produced by CsA in rats [54]. In addition, studies in animal models documented protective effect of pomegranate against a number of drug induced nephrotoxicity including gentamicin [55] and cisplatin [56].

Conclusion

This study demonstrates that the oral bioavailability of CsA is markedly increased after repeated concomitant administration with pomegranate juice . Independently, there is a protective effect of pomegranate juice against CsA induced kidney injury in rats. Future studies are warranted to determine the risk and benefit of this combination in human

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