Cyclosporine A Toxicity in Association With Reduced Renal Mass

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ABSTRACT

The long-term effect of cyclosporine A (CsA) in male Wistar rats with reduced renal mass was studied. The aim of the study was to highlight the relationship of CsA effect on rats, simulating patients with two functioning kidneys (eg, heart, liver transplant recipients) and one kidney (renal transplant recipients). The Wistar rats were subjected to unilateral nephrectomy (Unx, $n = 14$) and to 5/6 nephrectomy (STnx, $n = 14$). Half of these rats and half of the sham operated ones (control, $n = 13$) were administered CsA (10 mg/kg/d) for 28 days IP. The serum creatinine (S_{CR}), total protein (S_P), and urine protein (U_P) values as well as the whole blood CsA levels were determined on the 28th day of the study. The remnant kidneys were evaluated by image analyses and semiquantitative methods after sacrifice on the 28th day.

In the three non–CsA-treated groups (Unx, STnx, and control) S_{CR} was significantly higher in STnx rats than in Unx rats ($P = .011$). Percent of renal scarring (PRS) was significantly higher in Unx ($P = .02$) and in STnx rats ($P = .017$), compared with the control group. Among CsA-treated three groups S_{CR} was significantly higher in STnx rats compared with Unx ($P = .017$). In addition, segmental sclerosis rate (SSR) was higher in STnx rats, compared with the control group ($P = .008$), whereas S_P was higher in the control group ($P = .005$). When CsA-treated groups were compared with non–CsA-treated ones, U_P of the Unx rats not receiving CsA were significantly higher than the Unx rats receiving CsA ($P = .026$). Also, U_P was higher in non–CsA-treated groups ($P = .014$), whereas S_{CR} ($P = .001$), S_P ($P = .001$), and PRS ($P = .001$) were higher in CsA-treated rats.

In conclusion, we suggest that preserved renal mass is not enough to prevent CsA toxicity and that CsA should be administered to patients with both kidneys (eg, heart, pancreas recipients) as carefully as to patients with one functioning kidney (renal transplant recipients).

THE IMMUNOSUPPRESSIVE effects of cyclosporine A (CsA) in allograft rejection, particularly after kidney, liver, heart, and pancreas transplantation, have improved the survival of grafts markedly, since its introduction by Borel et al in 1976.[1] The most important side effects of CsA, occurring simultaneously with the immunosuppressive effects, are nephrotoxicity and hypertension.[2–4] A number of mechanisms have been described explaining the pathogenesis of toxicity caused by CsA, of which the most accepted one is the drug's interference with interleukin-2 gene transcription via a cyclosporine–cyclophilin complex inhibiting calcineurin phosphatase.[4,5] A renal transplant recipient is prone to reduced renal mass; this is not the case for other organ transplant recipients because they have both kidneys.

Reductions in renal mass lead to progressive focal-segmental glomerulosclerosis,[6] proteinuria followed by tubular atrophy, and interstitial fibrosis.[7–10] It is also reported that in rats undergoing extensive renal mass reduction, systemic hypertension and a decrease in both glomerular filtration rate (GFR) and renal blood flow (RBF) develop.[11]

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Renal mass reduction and CsA use, together, may lead to more severe renal damage or there might not be an additive effect. In the present study, it is aimed to investigate the extent of renal damage in unilaterally and subtotally nephrectomized rats receiving CsA, compared with the control group with two kidneys and to evaluate the changes in renal functions.

MATERIAL AND METHODS
Experimental Design

Groups of 41 male Wistar albino rats weighing 200–400 g were obtained. Twenty-eight were anesthetized with ether. Subsequent to a midline abdominal incision, 14 rats were unilaterally (right) nephrectomized (Unx) and in 14 rats, subtotal nephrectomy (STnx) was performed by right nephrectomy followed by partial infarction of approximately two-thirds of the left kidney by selective ligation of two to three of three to four extrarenal branches of the left renal artery.[12,13] Fourteen nephrectomized rats (7 Unx and 7 STnx) and 6 rats of the control group ($n = 13$) with two kidneys were given CsA for 28 days IP with a dose of 10 mg/kg/d (50 mg/mL, Sandimmun, Novartis Sağlık Gıda ve Tarım Ürünleri Sanayi ve Ticaret AŞ, Istanbul, Turkey).

Protocol

On the 28th day of the experiment, the rats were placed in individual metabolic cages for 24 hours. The 24-hour urine was used to determine proteinuria (U_P). Turbidimetric method was used to measure protein in urine (Hitachi 747 Autoanalyser, Tokyo, Japan). In addition, blood was obtained from the cut end of the tails of rats on the 28th day. CEDIA Cyclosporine PLUS assay (Microgenic, Fremont, USA) was used to measure CsA levels in whole blood (Hitachi 911 autoanalyser). CEDIA Cyclosporine PLUS assay uses DNA recombinant technology. The minimum detectable concentration of CEDIA Cyclosporine PLUS assay is 25 ng/mL. The high assay range quantifies 400–2000 ng/mL. Specimens quantitating greater than 2000 ng/mL diluted one part original sample with one part CsA free whole blood pretreated and reassayed. The value obtained on reassay derived as follows: actual value = 2 × diluted value. Serum total protein (S_P) and serum creatinine (S_{CR}) were measured using Biuret and Jaffe methods, respectively (Hitachi 747 Autoanalyser).[14] After 28 days of exposure to CsA intake, the rats were sacrificed and the remnant kidneys were removed.

Histopathologic Evaluation

For histologic examination, the kidneys were fixed in phosphate buffered 10% formalin. Sections (4 μm) were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and modified Masson's-trichrome (M-T) stains.

Quantification of Renal Scarring

For each slide, five standard areas (SA) were determined by drawing lines, extending from cortex to medulla, vertical to the capsule, dissecting the cut surfaces of the kidneys. Digital images obtained from the selected areas using light microscopy (Nikon, Labophot-2, Japan) at magnification ×10, by digital color video camera (Hitachi, WK-C220E) with software image analysis (Bs 200D Image Analysis Software, BAB Mühendislik Müh. Müş. San. Ve Tic. Ltd. Şti. Ankara, Turkey) were established and subse-
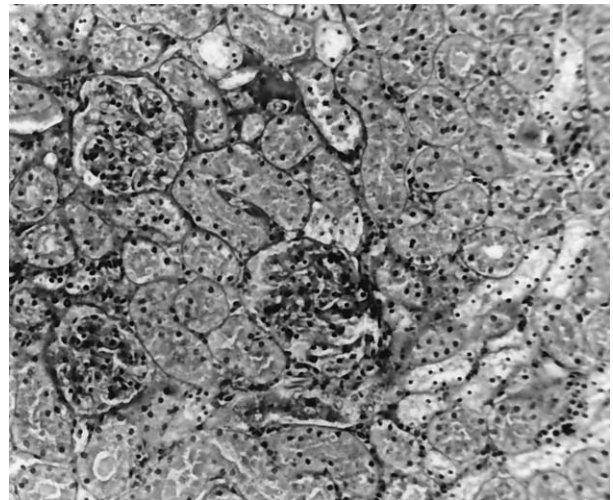
Fig 1. Of the three glomeruli, the one above is a normal-appearing glomerulus (0), whereas the ones below are mildly sclerotic (1+) (PAS, ×100).

quently these images were gathered in the computer (Vestel, Manisa, Turkey). Three cortical images in each SA and totally 15 images were evaluated for each rat. The total area was 6.05 mm^2.

For quantification of renal scarring, scarred renal areas as the indicator of chronic damage, were evaluated by a method slightly modified from the original description by Howie et al.[15] This includes global rather than segmental sclerotic glomeruli; areas of interstitial fibrosis, which appeared more solid and deeply stained than normal interstitial tissues; atrophic tubules, defined as tubules smaller than normal, with thickened basement membranes, or tubules larger than normal, with thin epithelium, including those large enough to be considered cysts; and arteries and arterioles, which are completely occluded.

With the image analysis system, using interactive area measurement method, interstitial scarred areas displaying the morphologic features described, have been recorded for every rat in 15 cortical images. The overall value was found as the total interstitial scarring area (T_{scar}) for every rat. The percentage of renal scarring (PRS) was determined by dividing the T_{scar} to the total test area.

Quantification of Glomerular Tuft Area

In these 15 cortical images, the cross-sectional area of approximately 50 glomeruli, outlined by Bowman's capsule, were measured again using the interactive area measurement method. The mean of approximately the largest 25% of areas was calculated (G_{area}).[16,17]

Quantification of Glomerular Sclerosis

Segmental sclerosis rate (SSR) was designated using the sections stained with H&E and PAS by light microscopy. Glomerular sclerosis is defined as an increase in mesangial matrix substance associated with capillary wall wrinkling and collapse,[18,19] and must be distinguished from the hyalinosis lesions, which are defined by the presence of PAS-positive hyalin material included or separated by sclerotic regions in the subendothelium or mesangium.[18] One hundred glomeruli were evaluated in renal sections of every rat. The method proposed by Wu et al[12] was used to determine the extent of glomerular injury. Each glomerulus was graded as either

Table 1. Mean Values of the Parameters Evaluated in Non–CsA-Treated Groups

	Unx ($n = 7$)	STnx ($n = 7$)	Control ($n = 7$)
U_P* (mg/kg)	0.024 ± 0.019	0.023 ± 0.015	0.018 ± 0.007
S_{CR} (mg/kg)	$0.89 \pm 0.19^£$	1.17 ± 0.11	0.71 ± 0.04
S_P* (mg/kg)	9.59 ± 4.48	9.34 ± 0.66	7.80 ± 0.26
G_{area}*	10124.14 ± 1018.07	12007.53 ± 3171.72	12054.68 ± 1742.88
PRS	$0.008 \pm 0.004^¶$	$0.062 \pm 0.10^§$	0.002 ± 0.001
SSR*	0.00 ± 0.00	0.86 ± 0.90	0.14 ± 0.38
Arteriol*	0.57 ± 0.53	0.71 ± 0.76	0.14 ± 0.38
Artery*	0.00 ± 0.00	0.43 ± 0.53	0.29 ± 0.49
T_{vac}*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Weight* (gr)	251.57 ± 25.09	191.71 ± 26.69	281.86 ± 38.86

All data are presented as mean $\pm$ SD.

Abbreviations: U_P, urinary protein; S_{CR}, serum creatinine; S_P, serum total protein; G_{area}, glomerular area; PRS, percent of renal scarring; SSR, segmental sclerosis rate; T_{vac}, tubular vacuolization.

*There was no statistical differences between any of the three groups ($P > 0.05$).

[£]Unx vs. STnx, $P = .011$;

[¶]Unx vs. control, $P = .002$;

[§]STnx vs. control, $P = .017$.

normal (0), mildly sclerotic (1+, lesion occupying less than 50% of glomerular tuft), severely sclerotic (2+, lesion occupying more than 50% of glomerular tuft) (Fig 1), or globally sclerotic (3+, lesion occupying 100% of glomerular tuft).

Evaluation of Vascular Lesions

Vascular lesions were evaluated according to the method proposed by Bertani et al,[20] as the presence of PAS positive material permeating the arteriolar wall and narrowing or occluding the vascular lumen or mucoid thickening of intima resulting in a narrowing of vascular lumens and were graded from 0 to 3+ (0 = no changes, 1+ = mild, 2+ = moderate, 3+ = severe).

Evaluation of Tubular Vacuolization

In addition, vacuolization, particularly in the proximal tubules, was semiquantitatively scored from 0 to 3+ (0 = no change, 1+ = mild change, 2+ = moderate change, 3+ = severe change).

Statistical Analysis

The data were analyzed by computer software (SPSS 10.0 Chicago, Ill, USA). The probability level of .05 or less was chosen to represent statistical significance. The results were assessed by Kruskal-Wallis, Mann-Whitney U, one-way ANOVA, and Spearman correlation tests. Spearman correlation coefficient (r) represented good correlation if it was greater than .50, moderate if it was between .25 and .50, no correlation if it was less than .25, and inverse correlation if it was negative.

RESULTS

Subtotal nephrectomy by selective ligation of two to three upper branches of the left renal artery caused infarction of about one half to two thirds of that kidney. There was no statistical differences in rat weights between any of the groups ($P < .05$). Whole blood CsA levels between groups receiving CsA were not also significantly different ($P < .05$). The mean values of the parameters evaluated in non–CsA-treated and CsA-treated groups are summarized in Tables 1 and 2, respectively.

In the three non–CsA-treated groups (Unx, STnx, and control) there was no significant difference in SSR between Unx and STnx groups ($P = .073$), whereas S_{CR} was signif-

Table 2. Mean Values of the Parameters Evaluated in CsA-Treated (10 mg/kg/d) Groups

	Unx ($n = 7$)	STnx ($n = 7$)	Control ($n = 6$)
U_P* (mg/kg)	0.009 ± 0.004	0.014 ± 0.009	0.014 ± 0.006
S_{CR} (mg/kg)	$0.96 \pm 0.28^£$	1.36 ± 0.23	1.08 ± 0.13
S_P (mg/kg)	10.53 ± 7.38	$9.06 \pm 2.09^¶$	14.90 ± 2.99
CsA* (ng/mL)	2076.00 ± 779.68	1638.86 ± 535.34	2155.67 ± 431.02
G_{area}*	11587.11 ± 2590.10	10628.62 ± 1228.04	8986.65 ± 1129.59
PRS*	0.015 ± 0.006	0.030 ± 0.06	0.011 ± 0.006
SSR	0.71 ± 0.76	$1.71 \pm 0.95^¶$	0.00 ± 0.00
Arteriol	$0.43 \pm 0.53^£$	$1.29 \pm 0.49^¶$	0.50 ± 0.55
Artery*	0.00 ± 0.00	0.43 ± 0.54	0.17 ± 0.41
T_{vac}*	0.00 ± 0.00	0.57 ± 0.79	0.17 ± 0.41
Weight* (gr)	292.57 ± 79.32	255.14 ± 97.89	200.67 ± 23.36

All data are presented as mean $\pm$ SD.

Abbreviations: U_P, urinary protein; S_{CR}, serum creatinine; S_P, serum total protein; CsA, whole blood cyclosporine level; G_{area}, glomerular area; PRS, percent of renal scarring; SSR, segmental sclerosis rate; T_{vac}, tubular vacuolization.

*There was no statistical differences between any of the three groups ($P > 0.05$).

[£]Unx vs. STnx, $P < .05$;

[¶]STnx vs. control, $P < .05$.

Table 3. Statistically Significant Parameters in Comparison of CsA-treated and non–CsA-treated Control Groups

	CsA treated ($n = 6$)	Non-CsA-treated ($n = 7$)	P
U_P (mg/kg)	0.012 ± 0.007	0.022 ± 0.014	.014
S_P (mg/kg)	14.90 ± 2.99	7.80 ± 0.26	.001
S_{CR} (mg/dL)	1.08 ± 0.13	0.71 ± 0.04	.001
G_{area}	8986.65 ± 1129.59	12054.68 ± 1742.88	.001
PRS	0.011 ± 0.006	0.0015 ± 0.0010	.001

Abbreviations: U_P, urinary protein; S_P, serum total protein; S_{CR}, serum creatinine; G_{area}, glomerular area; PRS, percent of renal scarring.

icantly higher in STnx rats than in Unx rats ($P = .011$). PRS was significantly higher in Unx ($P = .02$) and in STnx rats ($P = 0.017$), compared with the control group (Table 1).

Among the three CsA-treated groups (Unx, STnx, and control) both S_{CR} ($P = .017$) and arteriolar hyalinization and obliteration ($P = .026$) were significantly higher in STnx rats compared with Unx. In addition, SSR ($P = .008$) and arteriolar hyalinization and obliteration (with a marginal significance of $P = .051$), were higher in STnx rats, compared with the control group whereas S_P was higher in the control group ($P = .005$) (Table 2).

Thereafter, CsA-treated groups were compared with non–CsA-treated groups. U_P of the Unx rats not receiving CsA were significantly higher than the Unx rats receiving CsA ($P = .026$) while PRS value was significantly higher in the CsA treated Unx group ($P = .026$). CsA-treated control group had significantly higher S_P ($P = .001$), S_{CR} ($P = .001$), and PRS values ($P = .001$) when compared with the non–CsA-treated groups, however, U_p ($P = 0.014$) and G_{area} ($P = .001$) were higher in the non–CsA-treated groups (Table 3). There was no significant difference between CsA-treated and non–CsA-treated STnx groups for any of the parameters.

Unx rats, STnx rats, and the control group were compared with each other, without taking CsA intake into consideration (Table 4). There were statistical differences in PRS, SSR, arterial and arteriolar hyalinization and obliteration, S_{CR}, and rat weights between the groups. SSR

($P = .016$) and S_{CR} ($P = .00$) were found to be significantly higher, while arterial hyalinization and obliteration ($P = .056$) were higher with a marginal significance in STnx group compared with Unx rats. PRS was significantly higher in Unx group compared with control group ($P = .025$). On the other hand, SSR ($P = .002$), S_{CR} ($P = .00$), and arteriolar hyalinization and obliteration ($P = .014$) were significantly higher in STnx rats compared with the control group.

In CsA-treated groups, S_{CR} was directly correlated with arterial hyalinization and obliteration ($P = .035$, $r = .472$). S_P was inversely correlated with glomerulary area ($P = .042$, $r = .459$). In non–CsA-treated groups, U_P was inversely correlated with S_P ($P = .014$, $r = .528$). S_{CR} was directly correlated with PRS ($P = .045$, $r = .441$) and S_P ($P = .00$, $r = .715$).

SSR, S_{CR}, and U_P values were evaluated among six of the groups and there was no correlation between the individual groups. SSR was directly correlated with S_{CR} ($P = .001$, $r = .517$).

DISCUSSION

CsA has been used increasingly in renal transplant patients who have 50% or more decrease in their functioning renal mass. This brings up the important question of whether there are any changes in the toxic effects of CsA due to renal mass reduction. It is reported that renal mass reduction causes progressive segmental glomerular sclerosis, associated with progressive renal function disorder both in humans and experimental animals.

The only article about the effect of CsA on ablation nephropathy was introduced by Waldherr et al[6] suggested that CsA might facilitate early death due to uremia in rats with renal ablation. In this study, subtotally nephrectomized rats divided into four groups were administrated different doses of CsA; the control group received none. The effects of the drug were evaluated both by measuring the clinical-biochemical parameters and by observing the histopathologic changes using the light microscope. In the present

Table 4. Statistically Significant Parameters in Comparison of Unx, STnx, and the Control Groups (41 rats) Without Taking CsA Intake Into Consideration

	Unx	STnx	Control
U_P* (mg/kg)	0.02 ± 0.02	0.02 ± 0.01	0.02 ± 0.007
S_P* (mg/kg)	4.21 ± 3.21	3.91 ± 2.39	3.79 ± 1.67
S_{CR} (mg/dL)	0.92 ± 0.23[§]	1.26 ± 0.20[£]	0.88 ± 0.21
G_{area}*	$10,855.62 \pm 2037.37$	$11,318.07 \pm 2418.87$	$10,638.67 \pm 2141.19$
PRS	0.001 ± 0.006[¶]	0.05 ± 0.08	0.006 ± 0.006
SSR	0.36 ± 0.63[§]	1.29 ± 0.99[£]	0.08 ± 0.28
Arteriol	0.50 ± 0.52	1.00 ± 0.68[£]	0.31 ± 0.48
Artery	0.00 ± 0.00[§]	0.43 ± 0.51	0.23 ± 0.44
T_{vac}*	0.00 ± 0.00	0.29 ± 0.61	0.08 ± 0.28
Weight	272.07 ± 60.39[§]	223.43 ± 76.38	244.38 ± 52.51

*There was no statistical differences between any of the three groups ($P > .05$).
[§]Unx vs. STnx, $P < .05$;
[¶]Unx vs. control, $P < .05$;
[£]STnx vs. control, $P < .05$.

study, a control group of rats with two functioning kidneys were examined and the histopathologic changes have been evaluated by morphometric methods using image analysis, the results may be interpreted in continuation with the previous one.

In our study, we found increased PRS in CsA-treated rats with one or two kidneys, as expected. However, there was not a significant difference in subtotal nephrectomized rats. PRS was more extensive in Unx non–CsA-treated rats compared with the STnx non–CsA-treated ones and both STnx and Unx groups showed higher PRS compared with the control group with two kidneys. This was the case when all the groups (41 rats) were compared with a statistical significant result for Unx and control group rats ($P = .025$). Strikingly, there was not a significant difference in PRS for CsA-treated rats when STnx, Unx, or rats with both kidneys were considered. This points to the important impact of CsA in PRS, which is accepted as a prognosticator in many renal diseases.[15] In view of these statistical findings, we suggest that PRS is not related to renal mass if there is the impact of CsA. Supporting these results, S_{CR} was increased in cases with CsA treatment and was higher in STnx group compared with Unx group for cases not treated with CsA. Only a significant difference in S_{CR} was identified between STnx and Unx groups, but there was not a difference between these and the control cases with both kidneys in CsA-treated cases. These results also point to a shadowed effect of renal mass reduction on renal function in cases receiving CsA.

When the three groups (Unx, STnx, control) were compared, without taking CsA intake into consideration, PRS, SSR, arterial hyalinization and obliteration, and S_{CR} had statistically significant differences. PRS was significantly higher in Unx group and similarly SSR, S_{CR}, and arteriolar hyalinization and obliteration were significantly higher in STnx group, compared with the control group. When Unx rats were compared with STnx group, SSR, S_{CR}, and arterial hyalinization and obliteration were significantly higher in STnx group. These findings all bring us to a point that renal mass reduction is usually a sufficiently important factor in evaluating the toxic effects in especially the renal transplanted patients, whether or not the patient is receiving CsA.

There are a battery of functional and histopathologic parameters proposed as prognosticators for renal diseases, the most favored being interstitial fibrosis and tubular atrophy.[21] Recently, PRS has been proposed as an important prognosticator, and because it includes all interstitial, tubular, and glomerular scarring values, it seems to be the most valuable indicator.[15] Glomerular area is influenced by more than one factor in our experimental model. It is known that CsA reduces the glomerular blood flow through functional or structural decrease in renal flow, resulting in hypoplastic changes in glomeruli.[4,22,23] On the other hand, if there is loss of renal mass, the remnant glomeruli undergoes hypertrophy, known as compansatris hypertro-

phy.[24] Without considering any of the results of our study, we might have expected a decrease in glomerular area due to CsA treatment, but because there is a significant loss owing to reduced renal mass, there might as well be hypertrophy of the remnant renal mass. In our series, G_{area} was decreased for CsA-treated rats, probably as a result of the structural and functional CsA effect. As expected, G_{area} was decreased in CsA-treated rats with two kidneys, there was not a difference for cases with Unx and STnx groups. Segmental sclerosis, which might lead to glomerulosclerosis, is associated with loss of renal mass and it is accepted as a secondary form of the disease. It is suggested that the compensatory hypertrophied glomeruli are under the influence of increased oxygen radicals, which results in focal damage of glomerular tuft.[19] In our series, we could not find any difference in SSR when CsA-treated and non-treated groups were compared. This was also the case when non–CsA-treated groups were compared. However, we found increased SSR in CsA-treated rats for STnx group compared with the control group. This suggests that severely decreased renal mass is more prone to segmental sclerosis under the influence of CsA. However, this cannot be applied to cases with one kidney. Arteriolar lesions are an important CsA side effect on kidney.[4,25] Supporting the mechanisms underlying segmental sclerosis progression in the secondary form of the disease the statistical results for arteriolar lesions were similar to SSR results.

In the view of these findings, we suggest that preserved renal mass is not enough to prevent CsA toxicity and that CsA should be administrated to patients with both kidneys (heart, liver, pancreas and so on transplant recipients) as carefully as to patients with one functioning kidney (renal transplant recipients).

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