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Effects of Atorvastatin and Carvedilol on Chronic Cyclosporine Nephrotoxicity in Rats

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Abstract

Background: To evaluate possible renoprotective effects of carvedilol and atorvastatin in CsA induced kidney injury in a rat model.

Methods: Twenty-two rats were divided into five groups. Control, CsA, CsA+karvedilol, CsA+Atorvastatin, CsA +karvedilol+atorvastatin groups. Drugs were given for 21 days.

Results: Serum BUN and creatinine were significantly higher in the CsA group compared to control and were significantly lower in the CsA+carvedilol+atorvastatin group compared to CsA group. Tissue MDA levels were found to be lower in group 3, 4 and 5 than in CsA group. Tissue SOD in CsA +carvedilol and CsA+atorvastatin groups were found higher than the control and CsA groups. Tissue NO levels were found to be higher only in CsA+carvedilol+atorvastatin group compared to CsA group. Apical budding, hyaline casts and apoptosis in the tubular system was significantly higher in the CsA group than in the treatment groups. Osteopontin showed strong positivity especially in the CsA group. Osteopontin density was lower in the treatment group.

Conclusions: This is the first study to evaluate MDA, SOD and NO at tissue level for carvedilol in cyclosporine nephrotoxicity. Carvedilol and atorvastatin may contribute to the reduction of renal injury in chronic CsA nehropathy. These agents have provided a protective effect on renal functions especially in combined treatment.

Keywords: Cyclosporin nephrotoxicity; Carvedilol; Atorvastatin; Oxidative stress; Nitric oxide

Introduction

Cyclosporin A (CsA) is an immunosuppressive agent involved in the group of calcineurin inhibitors which are commonly used in management of transplantation and autoimmune diseases [1]. Despite new treatment strategies, it is still a widely used medication. However, numerous side effects restrict its usage in clinical practice; such as nephrotoxicity, neurotoxicity, hepatotoxicity, hypertension, dyslipidemia etc. Cyclosporin A might give rise to acute or chronic nephrotoxicity [1-9]. Acute form results from renal hemodynamic changes and is generally a reversible condition [7]. However, in setting of chronic, all compartments of the kidney manifest histological alterations which are permanent. Cyclosporin A induced chronic inflammation, oxidative stress and apoptosis result in progression of the damage and eventually chronic renal failure develops [3,4,6-12].

Currently, it has been shown that all patients using CsA longer than ten years develop chronic nephrotoxicity [6]. Hence, the identification of pharmacological approaches is important to improve long-term outcomes for retarding the progression of chronic nephropathy. Beside the dose reduction or cessation of the drug, a number of medications have been suggested to prevent the development of chronic CsA nephropathy, including calcium channel blockers, renin-angiotensin system (RAS) inhibitors, endothelin receptor blockers and nitric oxide (NO) precursors [2,7,13-17]. Studies in recent years confirmed that β blockers and statins have anti-oxidant, anti-fibrotic and anti-inflammatory properties other than their primary aim of use [18-29]. Therefore, one could assume that use of these medications might give favourable results also in chronic CsA nephropathy. However, there is no adequate data regarding their usage in this era [2,6].

Purpose of this study was to investigate possible renoprotective effects of carvedilol and atorvastatin in CsA induced kidney injury in a rat model; by using biochemical and histopathological examinations.

Materials and Methods

The study included 30 adult male Wistar albino rats, weighing 200-250 g, obtained from Experimental Animal Center of Gazi University Faculty of Medicine, Ankara, Turkey. The study protocol was approved by Local Experimental Animals Ethics Committee (0.05.06.00 / 94-4824).

Rats were kept in polycarbonate cages, with six rats per cage, at a temperature of 22-25°C and a humidity of 40-70%, with 12 h light and 12 h dark cycles. They were fed with standard chow and water. Drug administrations, blood samplings and weighing procedures were performed between 9:00 and 10:00 a.m. to minimize the circadian variations. During 24 h urine accumulation day (21th day of the experiment), they were kept individually in metabolic cages.

Experimental design and drugs

The rats were randomly allocated into five groups, included six rats in each group. Pharmaceutical forms of each drug were administrated to animals by subcutaneous route for 21 days. 2 ml of saline was administered to group 1; 25 mg/kg/day CsA was administered to group 2; 25 mg/kg/day CsA and 4 mg/kg/day carvedilol were administered to group 3; 25 mg/kg/day CsA and 10 mg/kg/day atorvastatin were administered to group 4; 25 mg/kg/day CsA, 4 mg/kg/day carvedilol and 10 mg/kg/day atorvastatin were administered to group 5. On day 22, all animals were anesthetized with 40 mg/kg intraperitoneal ketamine hydrochloride (Ketalar Flakon, 45 mg/kg, Pfizer, Istanbul, Turkey) and blood samples were obtained by intra-cardiac way for determination of renal functions. The subjects were sacrificed after the kidneys had been removed. Left kidney was stored at -80°C for enzymatic analysis and the right kidney was placed into 10% formalin solution for histopathological evaluation.

Blood urea nitrogen (BUN) and creatinine concentrations were used for determination of renal functions. During 21th day of the experiment, 24 h urine was collected and creatinine clearance (CrCl) was calculated using the following formula; CrCl (ml/min)=UCR × UV/sCr × 1440; where UCR shows 24 h urine creatinine, UV shows 24 h urine volume and sCr shows serum creatinine [30]. Serum and urine creatinine concentrations were measured by Jaffre method and BUN was measured by kinetic ultraviolet assay in an autoanalyzer.

For malondialdehyde (MDA) level, the kidney tissues were homogenized in 0.15 M potassium chloride solution and supernatant had been deproteinized. Then, the samples have been treated with thiobarbituric acid for color formation. Absorbances were measured at 535 nm by spectrophotometry and the results were calculated as nmol/g tissue [31]. For superoxide dismutase (SOD) activity, the renal tissues were homogenized in physiological saline; supernatant was measured after the centrifugation. Results were calculated as unit/g tissue, assuming 50% inhibition of activity as one unit enzyme activity [32]. For determination of Nitric oxide (NO) levels, renal tissues were homogenized in a buffer; then deproteinized by centrifugation and NO was studied from supernatant. Results were calculated as mol/g of tissue [33].

Histopathological analysis of the kidneys

An experienced pathologist who blinded to other study parameters examined the renal tissues processed with routine histological procedures.

Sections were stained with hematoxylin-eosin and analyzed for apical budding, hyaline silenders, interstitial vacuolization, apoptosis and calcification of tubules. It was considered whether these changes were positive or not. Masson Trichrome (Bio-Optica, 04-010802) staining was performed to evaluate the tubulointerstitial fibrosis and glomerular arteriolopathy on paraffin blocks. Periodic acid-Schiff (PAS) stain was applied to assess the basement membrane thickening (Bio-Optica, 130-802).

Osteopontin (OSP) (clones OP3N, mouse monoclonal IgG1, Novacastra) which is an extracellular matrix protein with 34 kilo Dalton in weight, was stained for immunohistochemical examination. Streptavidin-Biotin triple immunoperoxidase method was used to determine OSP expression and cytoplasmic staining for OSP was considered as positive in renal tubular epithelium. The intensity of staining was scored as +1 (weak), +2 (moderate) and +3 (high).

Statistical analysis

All statistical analyses were performed by using SPSS for Windows, Version 18.0 (Chicago, USA). The data were stated as the number of subjects or mean ± standard deviation. Mann-Whitney U and Kruskal-Wallis test have been applied to compare the two groups and the multiple groups, respectively. The chi-square test was used for comparison of categorical data. $P<0.05$ was considered as significant for all analyses.

Results

Kidney functions

Serum BUN concentrations were significantly higher in the CsA, CsA+carvedilol, CsA+atorvastatin and CsA+atorvastatin +carvedilol groups compared to that of controls on day 22 (respectively p: 0.02; p: 0.002; p: 0.002; p: 0.02) (**Table 1**). The BUN concentrations were lower in the treatment group compared to CsA group (**Table 1**).

However, statistical significance was observed only in the CsA +carvedilol+atorvastatin group (p=0.03) (**Table 1**). The lowest level was in the CsA+carvedilol+atorvastatin group when treatment groups compared with each other (p:0,01).

Serum creatinine level was significantly higher in the CsA group compared to the control group on day 22 (p=0.002). By

contrast, no significant changes were detected in the treatment groups compared to the control group (**Table 1**).

Table 1: Renal function parameters and their variability differences

	Control	Group 2	Group 3	Group 4	Group 5
BUN (mg/dl)	21.66 ± 5.31	53.00 ± 25.51&	33.83 ± 5.34&	37.33 ± 6.74&	27.66 ± 2.87€
Creatinin (mg/dl)	O.45 ± 0.02	0.50 ± 0.02&	0.46 ± 0.04	0.45 ± 0.05	0.44 ± 0.02€
Ccr (mg/dl)	1.02 ± 0.43	0.53 ± 0.10	0.69 ± 0.30	0.93 ± 0.42	0.95 ± 0.34€

Group 2: CsA; Group 3: CsA+Carvedilol; Group 4: CsA+Atorvastatin; Group 5: CsA+Carvedilol+Atorvastatin; &: Compared to control group p<0.05; € compared to CsA group p<0.05

Other three treatment groups had lower creatinine than CsA group. However, statistical significance was observed only in the CsA+carvedilol+atorvastatin group (p=0.03). There were no significant differences between treatment groups (**Table 1**).

Creatinine clearance was lower in all groups compared with the controls, but statistical significance was not observed (**Table 1**). It was lowest in the CsA group compared to treatment groups. Statistical significance, however, was found only in the CsA+carvedilol+atorvastatin group (p=0.02). There were no significant differences between treatment groups (**Table 1**).

Tissue MDA level: Tissue MDA levels were found to be higher in CsA group, CsA+atorvastatin and CsA+carvedilol groups than that of control group (p=0.002, p=0.01 and p=0.05, respectively). However, comparing to CsA group, tissue MDA levels were significantly lower in group 3, 4 and 5 (p=0.002; p=0.004 and p=0.004, respectively). The highest values were in CsA group and the lowest values were in the CsA+carvedilol group; inter-group differences were significant (p=0.005) (**Table 2**).

Table 2: Renal tissue MDA, SOD, NO levels and their variability differences.

	Control	Group 2	Group 3	Group 4	Group 5
MDA nmol/g	52.26 ± 6.63	88.53 ± 10.21&	46.82 ± 5.74€	66.45 ± 8.00€&	60.05 ± 7.96€
SOD U/g	599.99 ± 13.93	605.09 ± 27.52	633.12 ± 13.55&€	645.85 ± 18.63&€	613.58 ± 40.74
NO µmol/g	0.22 ± 0.05	0.12 ± 0.02&	0.15 ± 0.04&	0.13 ± 0.02&	0.21 ± 0.02€

Group 2: CsA; Group 3: CsA+Carvedilol; Group 4: CsA+Atorvastatin; Group 5: CsA+Carvedilol+Atorvastatin; &: Compared to control group p<0.05; €: Compared to CsA p<0.05

Tissue SOD level: Tissue SOD level of CsA group was similar to control group. However, CsA+carvedilol and CsA+atorvastatin groups had higher levels of SOD than that of control group (p=0.009 and p=0.002, respectively) (**Table 2**). Additively, CsA +carvedilol and CsA+atorvastatin groups had higher SOD levels compared to CsA group (p=0.04 and p=0.01, respectively). It was no significant difference between the treatment groups (**Table 2**).

Tissue NO level: Tissue NO levels were lower in CsA, CsA +carvedilol and CsA+atorvastatin groups compared to the control group (p=0.006, p=0.04 and p=0.004, respectively) (**Table 2**). Tissue NO levels were found to be higher only in CsA +carvedilol+atorvastatin group compared to CsA group (p=0.006). The highest levels of NO were observed in CsA +carvedilol+atorvastatin group among all treatment groups (p=0.01) (**Table 2**).

Histopathological analysis

There was not glomerular basement membrane thickening or particular pathological changes with hematoxylin-eosin and PAS staining in CsA group. Apical budding and hyaline casts in the tubular system was significantly higher in the CsA group than that of observed in treatment groups's (p=0.01 and p=0.04, respectively) (**Table 3**).

Table 3: Histopathological changes were compared over the groups.

	Apical budding		Hyaline cast	
	Yes	No	Yes	No
Control	0	6	1	5
Group 2	6	0	5	1
Group 3	1	5	2	3
Group 4	1	5	0	6
Group 5	1	5&	1	5€

Group 2: CsA, Group 3: CsA+Carvedilol, Group 4: CsA+Atorvastatin, Group: CsA+Carvedilol+Atorvastatin, &: Compare the five groups for apical budding p<0.05, €: Compare the five groups for hyaline casts p<0.05

Some samples of tubules revealed calcification in all groups, but it was not statistically significant. Extensive apoptosis, especially in CsA group, was remarkable in tubule epithelial cells. The treatment group had a substantial decrease in apoptosis. (p=0.006) (**Table 4**). Masson trichrome stain was used for the evaluation of interstitial fibrosis. Nevertheless, it was not detected in any samples. There were not interstitial vacuolization, wall thickening of arteries and arterioles, hyaline deposition, and lumen contraction. Osteopontin (OSP) showed strong positivity especially in the CsA group. Granular cytoplasmic staining of OSP was observed in tubules and medullary regions. OSP density was lower in the treatment group. Moreover, the OSP staining was different between

cyclosporine and the treatment groups and statistically significant (p=0.04) (**Table 4**).

Table 4: Histopathological changes and osteopontin staining were compared over the groups.

	Apoptosis		Osteopontin		
	Yes	No	Negative	Weak Positivity	Strong Positivity
Control	0	6	5	1	0
Group 2	4	2	0	2	4
Group 3	1	5	2	3	1
Group 4	0	6	2	3	1
Group 5	0	6€	4	2	0&

Group 2: CsA; Group 3: CsA+Carvedilol; Group 4: CsA+Atorvastatin; Group 5: CsA+Carvedilol+Atorvastatin; &: Compare the five groups for apoptosis p<0.01; €: Compare the five groups for osteopontin p<0.05

Discussion

In the presented study, we searched the potential renoprotective effects of carvedilol and atorvastatin in CsA induced kidney injury by using biochemical and histopathological alterations in a rat model. Our results showed that usage of CsA let to renal dysfunction, increased the tissue MDA levels and usage of carvedilol and atorvastatin reduced the functional disability.

CsA induced chronic ischemia begins with arteriolar vasoconstriction and subsequently proceeds with the hyalinosis. Intra-renal renin angiotensin system activation is an important mechanisms for CsA induced vasoconstriction [4,7,8,11,12,34-40]. Carvedilol is a potent vasodilator agent which blocks α1, β1, β2 adrenergic receptors and has anti-oxidant and anti-fibrotic effects. In a recent study, *in vitro* positive impacts of carvedilol on chronic nephrotoxicity have been shown [2,18,21,41]. We hypothesised that carvedilol might inhibit CsA-induced sympathetic activity and might suppress renin angiotensin system activity *via* β receptors. Our findings supported our hypothesis; carvedilol group alleviated biochemical and histopathological findings associated with CsA usage. It reduces the activity of angiotensin II, thereby renal blood flow and glomerular filtration rate are maintained [42,43]. However, the presence of apoptosis has been mainly recognized as a marker of chronic CsA nephrotoxicity in which studies with similar modeling [30,44]. As apoptosis is the precursor lesion, if the study had continued, it was likely to be followed by other histopathological findings, especially fibrosis.

The other aspect is a decrease in nitric oxide release in chronic CsA users, which increases mesangial cell proliferation, extracellular matrix protein synthesis, interstitial inflammatory cell infiltration and glomerular thrombosis [6]. *In-vitro* and *in-vivo* studies showed reduced glomerular and interstitial fibrosis through anti-fibrotic effects of endothelial synthase mediated NO release [2,45,46]. Carvedilol improves the endothelial functions, facilitates the release of nitric oxide and the other vasodilator agents [45,47] In our study, higher nitric oxide levels have been observed in carvedilol group, but the results did not reach statistical significance. We considered that the duration of carvedilol therapy might have influenced the increase in NO levels. Further studies with longer durations are warranted to support our results. Besides hemodynamic effects of NO, it has the anti-fibrotic and anti-inflammatory effects as well. In these studies, it has been suggested that NO may inhibit the formation of type 4 collagen, matrix proteins, plasminogen activator-1, α-smooth muscle actin and TGF-β1. NO, also has a preventive effect on macrophages infiltration that contributes to the antifibrotic activity [2,17,26,27,47,48].

Chronic ischemic process subsequent to chronic CsA usage increases reactive oxygen species in excess of the compensatory capacity for biological anti-oxidant molecules. By the way, increased oxidative stress causes renal cell damage, death through apoptosis, interstitial fibrosis and tubular atrophy [6]. Malondialdehyde is a product of lipid peroxidation and a well-known marker of oxidative stress. The use of carvedilol has been shown to provide anti-oxidant action by suppressing malondialdehyde levels [18-22]. In our study, we have shown decreased MDA levels in carvedilol plus CsA group. This group of rat also had increased tissue SOD level, an indicator of anti-oxidant activity. Furthermore, we showed a significant reduction in apoptosis in the carvedilol group. All these findings robustly support that carvedilol had favourable effect on oxidative stress and provided histopathological improvement. Hence, we consider that it might be protective in chronic CsA nephrotoxicity. Further studies are warranted. Wafaa et al. have conducted another study on cyclosporine nephroctosites related rats [2]. Çalışmalarında karvedilol gibi 3. Kuşak beta blokör olan nebivololün siklosporin nefrotoksistesi üzerine olan etkisini değerlendirmişlerdir. In their study, they evaluated the effect of nebivolol, the 3rd generation beta blocker, such as carvedilol, on cyclosporine nephrotoxicity. ADMA levels, an important indicator of increased inflammation and oxidative stress in the study, were measured in rat sera. In addition, ADMA is a NO synthase inhibitor. Therefore, elevated ADMA levels caused by cyclosporine may cause a decrease in NO levels. Wafaa et al. showed a significant decrease in ADMA levels *via* nebivolol in the results [2]. This conclusion and our conclusions support those third-generation beta-blockers such as carvedilol may have oxidative stress and inflammatory effects and elevated NO levels when evaluated together. Especially in our study, positive effects on oxidative stress and NO were shown at tissue level, making the results more reliable. In addition, our study is the first study to evaluate MDA, SOD and NO at tissue level for carvedilol for cyclosporine nephrotoxicity in the literature. Ultimately, these positive effects may have prevented the important mechanisms of cyclosporine nephrotoxicity [49].

Statins or 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors are drug of choice for lowering cholesterol. This class of drug have also pleiotropic effects such as regulation of endothelial function, NO synthesis, the reduction of oxidative stress and inflammation. In regard to kidney, previous data showed impaired mesangial and vascular smooth muscle cell proliferation, mesangial matrix expansion and macrophage infiltration by use of statins [50]. Therefore, statins could

provide additional benefit in preventing the fibrosis, interstitial inflammation and oxidative stress in setting of chronic CsA nephrotoxicity. There is limited data in literature at this point. Li et al., observed decreased inflammation and fibrosis by use of pravastatin added to cyclosporine in an experimental study [43]. Zhou Seng et al. have shown suppressed oxidative stress in renal tissue of rats by using statin [44]. In the present study, we have shown improved kidney functions and histopathological findings in atorvastatin group. Antioxidant effect is of great importance in the prevention of nephrotoxicity. Statin therapy has a further positive impact rising levels of NO in the renal tissue. Antioxidant effect is also proposed to be contributed to the NO bioavailability [2,43]. We have shown higher NO value in atorvastatin group however, there was no statistical significance, perhaps due to the short duration of treatment.

In setting of chronic CsA nephropathy, inflammatory process significantly contributes to fibrosis of renal tissue [5,6]. It has been showed that, osteopontin expression increases macrophage and monocyte chemotacsis; and eventually increases progression of renal fibrosis [2,5-7,12]. Therefore, osteopontin might have been suggested to be a pathogenic factor in CSA-induced renal injury [3,6,7,10,11]. In our study, intensity of osteopontin staining was lesser especially in atorvastatin group. Atorvastatin and carvedilol might be effective strategies in suppressing the OSP expreesion on tissue level.

We have shown that kidney function tests and histopathological findings were better in combination of carvedilol and atorvastatin group than the others. Besides their anti-adrenergic and anti-inflammatory effects, respectively, we considered that both have anti-oxidative function and inhibit osteopontin expression. They also increase bioavailability and synthesis of NO. As a result, the combined treatment could better protect kidney function biochemically with the favourable impacts on inflammation, oxidative stress, and NO synthesis, which will help to decrease apoptosis histopathologically.

Considering the weakness of our study, we thought that, short study time has prevented the emergence of complete histopathological findings. Secondly, the effects of carvedilol and atorvastatin on pro-fibrotic markers such as TGF-β1 and fibronectin have not been evaluated. Therefore, it is hard to interpret the effects of these treatments on fibrosis of renal tissue.

In conclusion, carvedilol and atorvastatin were found to be protective against the chronic CsA nephropathy both in the histological and biochemical evaluation in our study. These agents have provided a protective effect on renal function separately; the combined treatment was more pronounced as well. However, further *in vitro* and *in vivo* studies are needed to define the protective impact of the treatment. In addition, because of the multifactorial pathogenesis of chronic CsA nephrotoxicity, it could be considered that a single agent may not be adequate for a protective effect. Therefore, *in vivo* studies using combination treatments would provide more benefits.

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