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Effects of Angiotensin II Receptor Blockade on Soluble Klotho and Oxidative Stress in Calcineurin Inhibitor Nephrotoxicity in Rats

Sina Raeisi,[1,2,3] Amir Ghorbanihaghjo,[1] Hassan Argani,[4] Siavoush Dastmalchi,[5] Babollah Ghasemi,[6] Teimour Ghazizadeh,[2] Nadereh Rashtchizadeh,[5] Mahboob Nemati,[7] Mehran Mesgari Abbasi,[1] Nasrin Bargahi,[5] Ali Mota,[2] Amir Mansour Vatankhah[1]

[1]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Department of Biochemistry and Clinical Laboratories, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Urology and Nephrology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[5]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[6]Division of Clinical Laboratory, Tabriz Children's Hospital, Tabriz University of Medical Sciences, Tabriz, Iran
[7]Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

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Introduction. Calcineurin inhibitor nephrotoxicity is major problem after organ transplantation. It is multifactorial, but oxidative stress may have an important role in this process. It has been shown that angiotensin II receptor blockers have renoprotective effects but their molecular mechanism is largely unknown. Antioxidative effect is an important role of the recently known anti-aging protein, klotho. This study aimed to evaluate effect of valsartan in alleviation of cyclosporine A nephrotoxicity via a probable increase in serum klotho levels or decreasing oxidative stress.

Materials and Methods. Thirty-two Sprague-Dawley rats were divided into 4 groups to receive 1 mL/kg/d of olive oil as control; 30 mg/kg/d of cyclosporine; 30 mg/kg/d of cyclosporine and 50 mg/kg/d of valsartan; and 50 mg/kg/d of valsartan. After the 6 weeks of administration period, serum levels of klotho and 8-hydroxydeoxyguanosine were measured using an enzyme-linked immunosorbent assay. Serum malondialdehyde level was measured spectrophotometrically.

Results. The mean serum level of klotho was significantly lower in the cyclosporine group compared with control and valsartan groups. Klotho level in the valsartan group was significantly higher than those in the other groups. The cyclosporine group was detected to have significantly higher serum 8-hydroxydeoxyguanosine and malondialdehyde levels compared with the other study groups. The levels of klotho were negatively correlated with 8-hydroxydeoxyguanosine and malondialdehyde levels.

Conclusions. Administration of valsartan may lead to attenuation of the nephrotoxic side effect of cyclosporine via enhancing klotho and decreasing oxidative stress levels.

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INTRODUCTION

The introduction of the calcineurin inhibitor cyclosporine, as an immunosuppressive drug with excellent short-term outcomes in the late 1970s, revolutionarily improved transplantation medicine, and made it a preferable therapy for patients with end-stage renal disease.[1,2] Cyclosporine prevents calcineurin, a protein-phosphatase involved in the

T lymphocytes' activation, which leads to suppress the immune system. Cyclosporine alleviates the outcomes of organ transplantation, but its nephrotoxicity may lead to dramatic problems posttransplantation.[3,4] Although all mechanisms for cyclosporine-induced nephrotoxicity have not been known yet, some potential mediators such as prostaglandins, nitric oxide, endothelin, thromboxane, aldosterone, and renin-angiotensin system have been proposed as being responsible in this process.[1] According to the previous studies, oxidative stress as an alternate source of kidney damage has an important role in cyclosporine nephrotoxicity.[5-7]

There are some present and potential strategies to alleviate cyclosporine-induced nephrotoxicity.[1] Based on previous studies, valsartan, an antihypertensive drug, has also a renoprotective effect as documented by reduced urinary albumin and protein excretion in patients with diabetes mellitus or chronic kidney disease.[8-10] It belongs to the angiotensin receptor blockers, which block angiotensin II type 1 receptor. Angiotensin II type 1 blockade mediates blood pressure (BP) elevating effect of angiotensin.[11] Effects of different angiotensin receptor blockers on BP are close to each other, but some studies have shown that valsartan is the most specific, most effective, and safest drug of all.[11,12] It has also been shown that valsartan treatment does not interfere with immunosuppressive therapy.[12]

The molecular mechanisms responsible for the renoprotective effect of valsartan, independent from BP lowering, have not been clear yet.[13] Some studies have demonstrated the association of angiotensin receptor blockers in the elevation of klotho levels.[14-16] *Klotho* is a recently discovered anti-aging gene that is predominantly expressed in the kidneys.[17] This gene encodes a type I single-pass transmembrane protein. It also has a soluble secreted form that can be derived by alternative splicing or cleavage from membrane klotho. It can then circulate throughout the body fluids.[18] All roles of this protein have not been completely understood yet,[19] although it has become clear that one important role of soluble klotho is antioxidative effect[20,21]; thus, in a sufficient level, it may protect the kidneys from oxidative stress-inducing factors such as calcineurin inhibitors.

The aim of the present study was to evaluate valsartan effect in attenuation of cyclosporine nephrotoxicity via the probable increase in serum klotho and decreasing in oxidative stress levels.

MATERIALS AND METHODS

Animals

Thirty-two male 12-week-old Sprague-Dawley rats weighing 220 g to 280 g were purchased from Pasteur Institute of Iran (Tehran, Iran). According to the Guide for Care and Use of Laboratory Animals (DHEW Publication No [NIH] 78-23, revised 1978) and local guidelines for compassionate use of animals in research, they were housed 2 per cage, providing free access to tap water and compact standard chow. The animals were kept in similar laboratory conditions (18°C to 23°C room temperature and controlled humidity) with alternating 12-hour light and dark cycles.

Study Design

After a 2-week acclimation period, the weight-matched rats were randomly divided into 4 groups (8 rats per group): the control group (group A) received daily subcutaneous injections of vehicle (1 mL/kg of olive oil, Sigma). The cyclosporine group (group B) received daily subcutaneous injections of cyclosporine (Novartis) diluted in olive oil (15 mg/mL) at a dose of 30 mg/kg. Group C received both valsartan (Novartis Pharma; 50 mg/kg/d, in drinking water) and cyclosporine (30 mg/kg/d, subcutaneous injection). Group D received daily administrations of valsartan (50 mg/kg, in drinking water). The administration period was 6 weeks in all study groups. After the administration, all the rats were weighed. Afterwards, intravenous blood samples were taken, and after allowing for clotting, the sera were separated by centrifugation at 3000 g for 15 minutes and stored at -80˚C until the analyses were carried out.

Determining Serum Parameters

Serum concentrations of urea and creatinine were determined colorimetrically using commercial reagents in an automated chemical analyzer (Roche Cobas Mira). Furthermore, deteriorated kidney failure (DKF) index was calculated the sum of serum creatinine and urea divided by 2, as a way to better estimate the glomerular filtration rate (GFR). Malondialdehyde level, as a lipid peroxidation marker, was measured spectrophotometrically

using thiobarbituric acid reactive substances assay according to the method of Lapenna and colleagues.[22] Another marker of oxidative stress, 8-hydroxydeoxyguanosine (8-HDG), was measured by a rat enzyme-linked immunosorbent assay kit (Hangzhou Eastbiopharm, Hangzhou, China). Serum level of klotho was also determined by a rat enzyme-linked immunosorbent assay kit (Hangzhou Eastbiopharm, Hangzhou, China).

Statistical Analysis

Data are shown as mean ± standard deviation. Statistical comparisons of the groups were carried out by the 1-way analysis of variance and Bonferroni post-hoc analysis. The Pearson correlation coefficient was also calculated. A *P* value less than .05 was considered significant. The analyses were performed using the SPSS software (Statistical Package for the Social Sciences, version 16.0, SPSS Inc, Chicago, IL, USA).

RESULTS

Body and Kidney Weights and Serum Biochemical Parameters

Table 1 shows the basic parameters of the study groups after the administration period. All the rats were weight-matched before the intervention, but after the intervention, rats in group B (cyclosporine) had significantly lower mean body weight than the other groups ($P < .05$). The rats receiving cyclosporine (group B) were detected to have significantly higher serum urea, creatinine, and DKF index levels than the other study groups ($P < .05$).

Serum Levels of Klotho

As seen in Table 2, the mean serum level of klotho was significantly lower in the cyclosporine group compared with control and valsartan groups ($P < .05$). The valsartan group was detected to have significantly higher serum klotho levels than the other groups ($P < .05$). It is notable, there was not any significant difference in klotho levels between group C (cyclosporine and valsartan) and the controls.

Serum Levels of Oxidative Stress Markers

Serum levels of oxidative stress markers, 8-HDG, and malondialdehyde were measured and compared among study groups. As presented in Table 2, the rats receiving cyclosporine were detected to have significantly higher serum 8-HDG and malondialdehyde levels than the other study groups ($P < .05$).

Correlations of Klotho, Oxidative Stress, and Deteriorated Kidney Failure Index

As shown in Table 3, the serum level of klotho was negatively correlated with malondialdehyde and 8-HDG levels. These oxidative stress markers were positively correlated with each other. Klotho

Table 1. Biochemical Parameters in the Study Groups

Parameter	Group A (Control)	Group B (Cyclosporine)	Group C (Valsartan and Cyclosporine)	Group D (Valsartan)	*P*		
					Group B vs A	Group C vs B	Group D vs B
Body weight, g							
Before intervention	208.78 ± 12.58	207.14 ± 10.33	205.57 ± 6.00	210.11 ± 11.27	> .05	> .05	> .05
After intervention	235.00 ± 17.03	190.57 ± 19.72	223.71 ± 23.45	252.32 ± 20.23	< .001	.03	< .001
Serum creatinine, mg/dL	1.04 ± 0.22	1.98 ± 0.71	1.18 ± 0.65	0.82 ± 0.37	.005	.04	.001
Serum urea, mg/dL	59.44 ± 14.48	106.57 ± 37.77	70.86 ± 22.77	55.56 ± 13.70	.002	.04	.001
Deteriorated kidney failure index	30.24 ± 7.23	54.28 ± 19.12	36.02 ± 11.53	28.19 ± 6.96	.002	.04	.001

Table 2. Serum Levels of Klotho and Oxidative Stress Markers in the Study Groups

Parameter	Group A (Control)	Group B (Cyclosporine)	Group C (Valsartan and Cyclosporine)	Group D (Valsartan)	*P*		
					Group B vs A	Group C vs B	Group D vs B
Klotho, ng/mL	1.26 ± 0.42	0.66 ± 0.15 a	1.13 ± 0.23	1.80 ± 0.56*	.03	> .05	.001
Malondialdehyde, nmol/L	4.56 ± 1.61	7.96 ± 1.98 e	5.05 ± 1.38 f	4.26 ± 1.75 g e	.003	.02	.001
8-hydroxydeoxyguanosine, ng/mL	1.09 ± 0.44	1.97 ± 0.50 h	1.25 ± 0.57 i	0.85 ± 0.14 j	.002	.02	.001

*$P = .04$ vs group A and $P = .01$ vs group C

Table 3. Correlations Between Klotho, Oxidative Stress Markers and Deteriorated Kidney Failure Index

Parameter	Klotho		Malondialdehyde		8-Hydroxydeoxyguanosine		Deteriorated Kidney Failure Index	
	r	*P*	r	*P*	r	*P*	r	*P*
Klotho, ng/mL	…	…	-0.767	< .001	-0.703	< .001	-0.585	< .001
Malondialdehyde, nmol/L	-0.767	< .001	…	…	0.858	< .001	0.660	< .001
8-hydroxydeoxyguanosine, ng/mL	-0.703	< .001	0.858	< .001	…	…	0.715	< .001
Deteriorated kidney failure index	-0.585	< .001	0.660	< .001	0.715	< .001	…	…

level was inversely correlated with DKF index, but each of oxidative stress markers was positively correlated with DKF index.

DISSCUSION

We initially hypothesized that among the therapeutic strategies which may lead to alleviation of cyclosporine-induced nephrotoxicity,[1] valsartan might also lead to diminish the nephrotoxicity of cyclosporine, due to its proven renal protective effect.[8,9] It has been shown that valsartan treatment does not interfere with immunosuppressive therapy.[12] This drug can be therapeutically administered to patients with kidney diseases, as hypertension is a prominent cause and also outcome of kidney diseases.[23] Effects of different angiotensin receptor blockers on BP are close to each other, but some studies have shown that valsartan is the most specific, most effective, and safest drug of all.[11,12] These characteristics may make valsartan to be distinguished and unique among other agents alleviating cyclosporine nephrotoxicity.

In the present study, the serum levels of 8-HDG, as a direct indicator of oxidative DNA damage and malondialdehyde, as a factor determining the degree of lipid peroxidation, were significantly higher in cyclosporine group compared with the other study groups. This result can confirm the oxidative stress-inducing effect of cyclosporine. The elevation of reactive oxygen species generation by cyclosporine may be due to some effects of cyclosporine such as mitochondrial electron transport system inhibition and decreasing cellular antioxidant system. It may also be a result of cyclosporine metabolism by cytochrome p450.[24] Yoon and colleagues[25] in their study showed that cyclosporine administration could decline expression of the antioxidant enzymes manganese superoxide dismutase and hemeoxygenase-1 through changing in the expression of FoxO transcription factors. As another possible mechanism, O'Connell and colleagues[5] showed that thioredoxin interacting protein, an inhibitor of thioredoxin (an important reactive oxygen species scavenger), significantly increased following cyclosporine treatment.

In the present study, the results clearly revealed that cyclosporine administration decreased serum levels of klotho. Mitobe and coworkers[26] showed that oxidative stress could decrease klotho expression in a mouse kidney cell line. Our results showed that klotho was negatively correlated with 8-HDG, as well as with malondialdehyde. Thus, in the present study, a decrease in klotho level may be due to cyclosporine-induced oxidative stress. Although oxidative stress can decrease klotho level, if klotho can be upregulated or its reduction can be prevented, in a sufficient level, it may enhance oxidative stress resistance. Anti-oxidative effect is one of the important functions of soluble klotho which can play an important role in the protection of the kidney as the main klotho-expressing tissue. Yamamoto and coworkers[27] stated that soluble klotho could activate FoxO transcription factors via inhibiting insulin/insulin-like growth factor 1 signaling cascade. These transcription factors could upregulate the expression of manganese superoxide dismutase that could lead eventually to oxidative stress resistance.

In the present study, valsartan could diminish the effect of cyclosporine in both reduction of klotho level and enhancing oxidative stress. According to the results, serum levels of klotho in cyclosporine-treated rats were significantly lower in the cyclosporine group compared to the control and valsartan groups, but there was no significant difference between the group with cyclosporine and valsartan and the controls. Soluble klotho and oxidative stress markers were also negatively correlated. All the mechanisms of how valsartan can enhance klotho levels have not been clearly understood yet. Zhou and asociates[16] proposed that valsartan might downregulate the transforming

growth factor beta 1 and p53 gene expressions. The activity of specificity protein 1, as a transcription factor of klotho gene, might be inhibited by p53, whose expression could be increased by growth factor beta 1. Hence, valsartan may indirectly upregulate specificity protein 1 gene expression, eventually leading to increase the expression of the klotho gene. As another mechanism, since activation of the renin-angiotensin-aldosterone system reduces klotho gene expression,[28] valsartan through renin-angiotensin-aldosterone system blockade may upregulate klotho gene expression.

Effects of cyclosporine and subsequently valsartan treatment on kidney function were also evaluated in the present study. The DKF index, as a marker of kidney function, was significantly higher in the cyclosporine group compared to the other study groups. This result could confirm the nephrotoxic side effect of cyclosporine that might worsen kidney function, leading to nephropathy. It seems that the weight loss occurring in cyclosporine group might be due to nephropathy induced by cyclosporine. Moreover, Farthing and coworkers[29] suggested that cyclosporine might diminish appetite, particularly early during the treatment. cyclosporine has an effect on albumin metabolism which may include a reduction in synthesis, increased breakdown, or both.[29] It may also promote catabolism and this may be due to the decreased albumin and increased urea levels.

In the present study, the correlations of klotho with kidney function and oxidative stress markers were also evaluated. According to the respectively positive and negative correlations of DKF index with klotho and oxidative stress markers, valsartan alleviation of cyclosporine-induced renal damage might be mediated by enhancing klotho and subsequently decreasing oxidative stress levels. Anti-oxidative effect of klotho is due to its soluble form that circulates throughout the body fluid,[18,19,27] and it can be measured in the serum samples. Serum levels of klotho, as a derived form of membrane klotho, can be an indicator of its expression level. Therefore, molecular study (renal klotho expression) was not done in the present study. However, it could be a limitation of the study. Blood pressure and hydration status measurements were not done in the present study and they could be other limitations of the study.

CONCLUSIONS

This study demonstrated that cyclosporine could lead to decreased klotho levels, possibly through enhancing oxidative stress. Valsartan treatment might ameliorate cyclosporine-induced oxidative stress via increasing klotho level that eventually lead to alleviation of cyclosporine nephrotoxicity and kidney function improvement.

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CONFLICTS OF INTEREST

None declared.

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Correspondence to:
Amir Ghorbanihaghjo, PhD
Drug Applied Research Center, Tabriz University of Medical Sciences, Daneshgah St, Tabriz, PO Box 51666, Iran
Tel: +98 413 381 4965
Fax: +98 413 442 6078
E-mail: ghorbaniamir@hotmail.com

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