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Differential Status of Serum Arginine, Arginase and Nitric Oxide in Patients of Chronic and Advanced Stage Kidney Disease Undergoing Hemodialysis

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Chronic kidney disease (CKD) is characterized by deterioration of endothelial function which is associated with reduced availability of nitric oxide. The objective of the study was to assess the differential status of the serum levels of arginine, arginase, NO, urea and creatinine in CKD patients not on hemodialysis and in end stage renal disease (ESRD) patients receiving hemodialysis. In this case control study, clinically diagnosed 30 CKD patients (group I), 30 ESRD patients before hemodialysis (Group II), 30 patients with ESRD after first hemodialysis (group III) and 30 patients with ESRD after second hemodialysis (Group IV) were included. 30 healthy volunteers were included for comparison. Serum arginine, arginase, nitric oxide, urea and creatinine were estimated by colorimetric and spectrophotometric methods. Serum creatinine and urea levels were evaluated to determine the severity of renal dysfunction. A significantly decreased serum arginine and nitric oxide levels whereas significantly increased serum arginase levels were observed in ESRD patients when compared to levels in CKD patients. Serum creatinine levels were significantly decreased after second hemodialysis. But there was no significant change in the serum levels of arginine, arginase and nitric oxide in ESRD patients after first and second hemodialysis compared to pre-dialysis group. The findings of the study throw light upon the differential status of serum arginine, arginase and nitric oxide in CKD and in ESRD patients. The evaluation of decreased nitric oxide levels coupled with elevated arginase activity may help in assessing progression of CKD to ESRD along with traditional markers of kidney function. Additionally, evaluation of serum arginase activity may provide useful prognostic information, with large study group and further follow-up, in hemodialysis patients.

Keywords: Arginase; Arginine; Nitric oxide; CKD; Hemodialysis.

Chronic kidney disease (CKD) is a gradual loss of kidney function may be over months to years of time period. It comprises of many different pathological and physiological factors associated with the abnormal functioning of the kidney and a progressive fall in the ability of kidney to properly clear waste products thus leading to a diminution of glomerular filtration rate (GFR). As the function of kidney declines, the number of nephrons also reduces in the advanced CKD stages[1]. Eventually dialysis has to be implemented to prevent azotemia which may amount to organ damage and death[1, 2]. The pathophysiology behind CKD can be due to genetic or developmental

abnormalities. There is also contribution of certain risk factors like hypertension, diabetes mellitus, hyperhomocysteinaemia, autoimmune diseases, inflammation, older age and previous insult by an episode of acute kidney disease which deteriorate kidney function[3]. With chronic insult, the over-all renal blood flow declines as a sequel of arteriolar vasculopathy, vascular blockage and reduced vascular mass. As the nephron number and function collapses there is maladaptive hypertrophy and sclerosis of nephron. This leads to reduction in renal mass over the years[2,3]. The CKD usually remain underdiagnosed and undertreated, leading to missed opportunities for prevention of its deterioration. This results in permanent dependency on therapies that replaces renal function like dialysis or kidney transplant[4].

The commonly accepted criteria to initiate hemodialysis include unresponsiveness to hyperkalemia, refractory acidosis, GFR below 10ml/min per 1.73 m^2, persistent extravascular volume expansion, etc. In clinical practice, creatinine clearance is typically used to assess renal function[2].

Patients with CKD show characteristic changes in kidney and its vasculature which includes endothelial dysfunction and progressive decrease in GFR[5]. The drop in GFR results from diminution of renal flow caused by vasoconstriction of renal vasculature. Normally, the endothelium produces many molecules responsible for vasodilatation, nitric oxide (NO) being the central[5]. Endothelium-derived nitric oxide has been seen to contribute to the regulation of regional blood flow by controlling the vascular tone[6,7]. Inhibition of endothelium-derived nitric oxide formation increases blood pressure and vascular resistance[8]. NO is synthesized from L-arginine by the action of enzyme, nitric oxide synthases (NOSs). In the kidney, NO takes part in several essential processes namely the maintenance of glomerular and medullary hemodynamics, regulation of the tubuloglomerular feedback response, extracellular fluid volume maintenance and renin release[6,8,9]. The deterioration of endothelial function is due to reduced availability of NO which may be due to reasons like rise in inhibitors of nitric oxide synthase, increased activity of arginase, decrease availability of arginine as a substrate or degradation of NO by oxygen radicals[9,10]. Arginine is also metabolized by arginase to form ornithine and urea in equimolar concentration. Additionally, L-arginine participates in the synthesis of nitric oxide within the kidney endothelium. So, the availability of arginine decides the level of NO in the endothelium. NO plays a key role in regulating the renal function through the maintenance of renal blood flow, blood pressure, vascular tone modulation and sodium balance[11,12]. Previous studies have investigated the serum arginine and associated metabolites in chronic renal disease with diminished levels of renal function[1,3,8,9,10]. However, there is paucity of reports on differential status of serum levels of these parameters in CKD patients not on hemodialysis and in patients with advanced stage of the CKD i.e. end stage renal disease (ESRD) before and after successive hemodialysis. Hence, considering altered arginine metabolism and its effect on kidney function, the present study was carried out to comparatively measure the serum levels of arginine, arginase, NO, urea and creatinine in CKD patients not on hemodialysis and in advanced stage CKD patients (ESRD patients) and to evaluate their role in assessing progression of the disease. Additionally, we comparatively evaluated the effect of successive hemodialysis on serum levels of these parameters in advanced stage of CKD (ESRD) patients.

MATERIAL AND METHODS

In this case control study, carried out from October 2013 – October 2015, in the Department of Biochemistry, B. J. Govt. Medical College, Pune, 120 clinically diagnosed patients of CKD and those with advanced stage of CKD (ESRD) undergoing successive hemodialysis, confirmed with the help of renal function test i.e. creatinine and blood urea irrespective of age & gender were included. Severity of CKD was assessed by renal function test i.e. creatinine and urea. Clinically relevant CKD was marked by functional abnormalities of the kidney or structural kidney damage for $\geq$ 3 months accompanied by reduced or no change in GFR. On the basis of severity, patients were further arranged into four groups. Group I included 30 cases of chronic kidney disease but not on hemodialysis. Group II included 30 cases with advanced CKD (ESRD) before first hemodialysis (pre-dialysis group). Group III included 30 cases with advanced

CKD (ESRD) after first hemodialysis and Group IV included 30 cases with advanced CKD after second hemodialysis. 30 healthy volunteers were included for comparison.

Inclusion criteria

Patients clinically diagnosed as chronic kidney disease not undergoing dialysis and those with advanced CKD or ESRD undergoing first and second hemodialysis, were included and confirmed with the help of renal function test.

Exclusion Criteria

Patients with acute kidney failure, drug induced renal disease, congestive heart failure, diabetes, smoking and current pregnancy were excluded from the study to avoid false results.

The institutional ethical committee approved [Reference number: BJMC/IEC/Pharmac/D-1113158-158] the study. Informed written consent was taken and 3 ml venous blood samples were collected from healthy volunteers and CKD patients of four study groups. The anti-cubital venous blood samples were taken after all aseptic precautions using sterile needles and syringes. Samples were allowed to clot at room temperature in a clean dry sterile plain bulb for 45 min and then centrifuged for 15 minutes at 2500 rpm. Serum was stored in two separate aliquots at -80^{o}C until analysis. Separated serum was used for estimation of arginine, arginase, nitric oxide, creatinine and urea.

Estimation of serum arginine

Serum arginine levels were estimated by Sakaguchi's method[13]. Briefly, to 0.5 ml serum 2.5 ml D.W., 0.5 ml 10% KOH and 1.0 ml 8-hydroxyquinoline was mixed and the test tubes were kept in ice bath for 10 min. Then, the tubes were removed and 0.5 ml freshly prepared sodium hypobromite solution was added followed by 0.5 ml urea solution. The red colour developed was measured immediately at 530 nm. From standard graph of arginine, the concentration of serum arginine was determined.

Estimation of serum arginase

Serum arginase levels were estimated by Roman and Ray method[14]. Briefly, 50 µl of serum was added to 250µl of $MnCl_2$ and the contents were incubated at 37^{0}C for 5 minutes. 500 µl of buffered substrate was added. Contents were mixed and incubated at 37^{0}C for 20 minutes. 2 ml of color developer was then added and contents were incubated at 95^{0}C for 15 minutes. The contents were cooled under tap water and then read at 530nm against blank treated similarly. The arginase concentration of sample was determined by using the standard curve of arginase [normal range upto 4IU/L].

Estimation of serum nitric oxide

Serum nitric oxide levels were measured by cadmium reduction spectrophotometric method[15]. Deproteinization of the serum sample was done by Somogyi reagent. 0.5ml of serum was mixed with 2ml with 75mmol/L $ZnSO_4$ solution kept for 10 minutes and then centrifuged. Then, 1ml of glycine-NaOH buffer was mixed with 1 ml of deproteinized sample. Then 3ml of deionized water was added followed by 2.5gm of activated cadmium granules. The mixture was then swirled and incubated for 90 minutes. 2 ml of from this mixture was then added with 1ml of sulphanilamide and 1ml of N-naphthylethylene diamine. After mixing, the tubes were covered with silver foil for 20 minutes and were read at 545nm. Serum nitric oxide concentrations were calculated using standard curve of nitric oxide (10-100µmol/L).

Estimation of Urea

Serum urea was estimated by kinetic enzymatic GLDH method by using commercially available kit[16]. Briefly 1000µl of working urea reagent was mixed with 20µl of serum. The rate of change in absorbance at 340nm is directly proportional to the urea concentration in serum. The standard was treated similarly and color intensities were compared. The urea concentration was calculated using standard formula using change in absorbance of sample and standard.

Estimation of creatinine

Serum creatinine concentration was estimated by using Modified Jaffe's method[17] using commercially available kit. Briefly, 1000µl of working creatinine reagent was mixed with 100 µl of serum. Standard was also treated similarly. Contents were mixed and the rate of change in absorbance of sample and standard was recorded. The creatinine concentration was calculated using standard formula.

Statistical analysis

The biochemical data was expressed in terms of mean ± SD. The significance in the outcome between healthy controls and CKD patients (group I) as well as CKD patients (group I) and pre-dialysis advanced chronic kidney

disease (ESRD) patients (group II) was statistically analysed by using Student's t test (unpaired). The significance of the outcomes among patients with advanced CKD (ESRD) before and after first and second hemodialysis was statistically analysed by using paired sample 't' test. Results with P<0.05 were considered statistically significant. The data was analysed using MedCalc statistical software.

RESULTS

The mean age of distribution was 50.9 years in control group, 52.2 years in Group I (CKD) and 50.2 years in Group II (ESRD) which shows that mean age of distribution was nearly equal in all the three study groups (F value= 0.50, P=0.61). The control group included 19 males and 11 females, Group I (CKD) included 18 males and 12 females and group II (ESRD) included 19 males and 11 females suggesting gender distribution was nearly equal in all the groups (Chi square= 0.95, P>0.05).

The serum levels and statistical comparisons of arginine, arginase, nitric oxide, urea and creatinine in healthy controls and CKD patients (group I), healthy controls and advanced CKD (ESRD) patients (group II) and CKD patients (group I) and ESRD patients (group II) are presented in table 1, 2 and 3 respectively. In the present study, significantly reduced serum arginine and nitric oxide and significantly higher serum arginase levels were observed CKD as well as in ESRD patients as compared to levels in healthy controls (P<0.0001). Serum urea and creatinine concentrations were significantly higher in patients with chronic kidney disease as well as in ESRD patients as compare to healthy controls (P<0.0001). Additionally, we observed significantly decreased serum arginine (P=0.0033) and nitric oxide (P<0.0001) levels and

Table 1. Depicts serum levels of arginine, arginase, nitric oxide, urea and creatinine in healthy controls and CKD patients (Group I)

Group	Healthy controls	CKD [Group I]	P value
Arginine (µmol/L)	71 ± 7.58	49 ± 6.61	P<0.0001
Arginase (IU/L)	2.37 ± 0.71	9.61 ± 2.69	P<0.0001
Nitric oxide (µmol/L)	61.63 ± 7.18	43.03 ± 6.85	P<0.0001
Urea (mg/dl)	21.46 ± 4.93	85.56 ± 16.17	P<0.0001
Creatinine (mg/dl)	0.87 ± 0.19	2.66 ± 0.82	P<0.0001

Table 2. Depicts serum levels of arginine, arginase, nitric oxide, urea and creatinine in healthy controls and advanced stage renal disease (ESRD) patients (Group II)

Group	Healthy controls	ESRD [Group II]	P value
Arginine (µmol/L)	71 ± 7.58	44.75 ± 3.72	P<0.0001
Arginase (IU/L)	2.37 ± 0.71	26.92 ± 4.98	P<0.0001
Nitric oxide (µmol/L)	61.63 ± 7.18	31.9 ± 7.03	P<0.0001
Urea (mg/dl)	21.46 ± 4.93	187.6 ± 38.8	P<0.0001
Creatinine (mg/dl)	0.87 ± 0.19	5.50 ± 2.01	P<0.0001

Table 3. Depicts serum levels of arginine, arginase, nitric oxide, urea and creatinine in CKD patients (Group I) and advanced stage renal disease (ESRD) patients (Group II)

Group	CKD [Group I]	ESRD [Group II]	P value
Arginine (µmol/L)	49 ± 6.61	44.75 ± 3.72	P=0.0033
Arginase (IU/L)	9.61 ± 2.69	26.92 ± 4.98	P<0.0001
Nitric oxide (µmol/L)	43.03 ± 6.85	31.9 ± 7.03	P<0.0001
Urea (mg/dl)	85.56 ± 16.17	187.6 ± 38.8	P<0.0001
Creatinine (mg/dl)	2.66 ± 0.82	5.50 ± 2.01	P<0.0001

Table 4. Depicts serum levels of arginine, arginase, nitric oxide, urea and creatinine in advanced stage renal disease patients (ESRD) before and after hemodialysis

Parameter	Advanced Stage Renal Disease (ESRD)		
	Before hemodialysis [Group II]	After first hemodialysis [Group III]	After second hemodialysis [Group IV]
Arginine (µmol/L)	44.75 ± 3.72	46 ± 4.98	45.96 ± 3.88
Arginase (IU/L)	26.92 ± 4.98	26.1 ± 3.53	24.75 ± 3.45
Nitric oxide (µmol/L)	31.9 ± 7.03	34.23 ± 6.59	33.66 ± 7.95
Urea (mg/dl)	187.6 ± 38.8	186.8 ± 26.84	184.93 ± 30.2
Creatinine (mg/dl)	5.50 ± 2.01	6.10 ± 2.54	4.29 ± 1.95

Table 5. Statistical analysis of arginine metabolites and creatinine in advanced stage renal disease patients (ESRD) before and after first and second hemodialysis

Parameter	Group II Vs Group III	Group II Vs Group IV	Group III Vs Group IV
Arginine	P= 0.32	P= 0.22	P= 0.97
Arginase	P= 0.32	P= 0.0548	P= 0.14
Nitric oxide	P= 0.16	P= 0.36	P= 0.76
Urea	P= 0.93	P= 0.76	P= 0.79
Creatinine	P= 0.31	P= 0.0213	P= 0.003

significantly increased serum arginase (P<0.0001), urea (P<0.0001) and creatinine (P<0.0001) levels in ESRD patients as compared to levels in CKD patients. The serum levels of arginine, arginase, nitric oxide, urea and creatinine in patients with advanced stage renal disease (ESRD) before hemodialysis (group II) and after first (group III) and second (group IV) hemodialysis are depicted in table 4. No significant change in the serum levels of arginine, arginase, nitric oxide and urea were observed in ESRD patients after first and second hemodialysis as compare to levels before hemodialysis (table 5) as well as in patients after undergoing second hemodialysis as compare to their levels after first hemodialysis. However, interestingly, serum arginase levels were only marginally statistically indifferent (P=0.0548) after second hemodialysis when before hemodialysis levels were compared. The serum creatinine levels were not significantly different after first hemodialysis when before hemodialysis (P=0.31) levels were compared. However, the serum creatinine levels were significantly decreased after second hemodialysis as compare to levels before hemodialysis (P=0.0213) as well as levels after undergoing first hemodialysis (P=0.003).

DISCUSSION

In this study, we have shown differential status of serum levels of arginine, arginase and nitric oxide along with kidney function test parameters including urea and creatinine in patients with CKD not on hemodialysis and in advanced CKD (ESRD) patients and compared them with healthy controls. Additionally, we measured the outcome of first and second hemodialysis on serum level of above parameters and compared them with ESRD patients before hemodialysis.

There is increasing documentation which states that renal vasculopathy or renal microangiopathy are precipitating determinant in the injury mechanism that over the time will thrust gradual damage to renal vasculature. The clinical progression of CKD has been shown to be preceded by endothelial dysfunction[2,18]. Under the physiological conditions, the intact endothelium is responsible for releasing of an important vasodialator known as NO. It is produced from amino acid arginine (which is endogenously synthesized in kidney). Arginine acts as a substrate for both NOS and arginase simultaneously. When catalysed by NOS it forms NO and citrulline.

And when acted upon by arginase produces urea and ornithine in equimolar concentration [9, 10, 19]. NO which is an essential molecule, involved in various physiological functions such as vasodilatation (anti-hypertensive) and anti-aggregation of platelets, inhibition of migration and proliferation of vascular smooth muscle cell and reduces the vascular production of superoxide radicals[10]. Many studies have reported that patients suffering from CKD have reduced availability of NO[9, 10, 20]. Reddy YS, et al[21] reported significantly lower plasma NO levels in different stages of CKD compared to controls and reduced plasma arginine in stage 5 of CKD. Wever R, et al[22] reported decreased NO production in chronic kidney failure patients not on hemodialysis. El-Sadek et al[8] reported significantly decreased serum arginine levels in pediatric patients with CKD. Our observations were in agreement with their reports[8, 21, 22]. However, in contrast to our reports, Azouaou LT, et al[3] reported significantly higher levels of serum nitric oxide in different stages of CKD as compared to controls. CKD is characterized by increase in production of free radicals and oxidants which also continues the role in progression of disease. The increased oxidative stress causes diversion or utilization of O_2 in production of superoxide ($O_2^{\cdot-}$) radicals. This leads to decreased availability of oxygen required for NO production by NOS isoforms. Hence, oxidative stress causes decreased NO production in CKD patients[9, 10]. The net NO deficiency in CKD may also be associated with decreased L-arginine synthesis by kidneys, elevated levels of ADMA, a potent NOS inhibitor and/or utilization of L-arginine by arginase involving metabolic pathways. Compromised conveyance of L-arginine to NOS may additionally occur due to defective transport in endothelium and/or decreased NOS activity due to changes in phosphorylation or lack of essential cofactors[9]. The chronic inhibition of NOS may induce increase in blood pressure and development of the scar tissue in the glomeruli (FSGS), the hallmarks of developing CKD[10]. Considering the constant oxidative stress generated in the initial stages of CKD, associated decreased nitric oxide synthesis and CKD linked impaired NO production contributes to gradual kidney damage and development of ESRD after a period of time[9, 10]. To our knowledge, we reported the comparison of serum arginase in controls, in CKD patients and in pre-dialysis ESRD patients for the first time. Earlier reports by Razmi NA et al[23] have reported effect of hemodialysis on serum arginase activity and compared it with pre-dialysis group. The decreased levels of arginine and nitric oxide and elevated serum urea and creatinine in ESRD patients were reported previously by Chris B et al[9,10]. Our findings are in agreement with their reports. The reduced availability of NO may be due to reduced availability of arginine or inhibition of NOS which in turn increases the availability of arginine for arginase[9,10]. The significantly elevated serum arginase and significantly reduced serum nitric oxide in ESRD patients before hemodialysis as compared to CKD patients not on hemodialysis provides a new insight on clinical utility of these markers in assessing the severity and progression of the disease from early-stage CKD to ESRD, in addition to traditional markers used to assess kidney function, serum creatinine and urea. This is because, commonly used markers for estimation of nephron function are creatinine and cystatin C. However, there is a controversy regarding sensitivity of creatinine in literature. Creatinine underestimates the condition (as it also originates from muscle wasting). And cystatin C is not cost effective [24,25]. Therefore, these new markers, serum arginase and nitric oxide, could potentially help to alarm us regarding progression of the disease at an early stage. In addition to above findings, we also studied effect of first and second hemodialysis on serum levels of arginine, arginase and nitric oxide along with serum urea and creatinine. Ugurcu V, et al[26] reported no significant change in serum arginine and nitric oxide levels in dialysis patients when control group levels were compared. Duranton F et al[27] observed significantly reduced plasma arginine levels in hemodialysis patients when values were compared to patients with CKD stage 2 to 5. Meenakshi SR, et al[1] Kovacevic et al[7], however, reported increased nitric oxide levels in chronic renal failure patients after hemodialysis when compared to control group. Tektas et al[28] reported increased serum arginase activity and decreased nitric oxide metabolites in hemodialysis patients compared to control group. In our study, the serum arginase activity after first hemodialysis was not statistically significant as compared to pre-dialysis ESRD group. The P value for serum arginase after second hemodialysis compared to pre-dialysis

group was slightly greater than the level of significance (P=0.0548) making this association statistically not significant, but this may be due to the small number of patients in our study group. So, further studies with larger sample size and follow-up is required to assess the clinical usefulness of arginase as a prognostic marker in these patients. On the contrary, creatinine-a metabolic waste product, is removed by dialysis causing significant decrease in its levels after hemodialysis. Increased arginase activity is reported to promotes eNOS-uncoupling, oxidative stress and inflammation ultimately leading to vascular disease[29]. So, we postulate that higher activity of arginase may contribute to the development of vascular diseases in hemodialysis patients due to vascular oxidative stress and inflammation. And therefore, we propose arginase as therapeutic target for prevention of vascular complications in hemodialysis patients.

CONCLUSION

The findings of the present study provide new insights on the differential status of serum arginine, arginase and nitric oxide in CKD and in ESRD patients undergoing hemodialysis. The evaluation of decreased nitric oxide levels coupled with elevated arginase activity may help clinicians in assessing progression of CKD to ESRD along with traditional markers of kidney function. Additionally, evaluation of serum arginase may provide useful prognostic information with large study group and further follow-up, in hemodialysis patients and provides background for further studies to explore arginase as therapeutic target to prevent vascular complications in hemodialysis patients.

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Conflict of Interest

There are no conflict of interest.

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