Evaluation of Plasma Concentrations of Homocysteine, IL-6, TNF-alpha, hs-CRP, and Total Antioxidant Capacity in Patients with End-Stage Renal Failure

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Abstract- It has been proved that hyperhomocysteinemia has a high prevalence in patients with end-stage renal disease (ESRD), which may contribute to the high cardiovascular risk in these patients. Cardiovascular disease is the first cause of high mortality rate in ESRD patients. The aim of the present study was to assess five important factors in patients with ESRD (the amount of homocysteine, IL-6, TNF-alpha, hs-CRP, and Total Antioxidant Capacity). These factors were surveyed in ESRD patients to compare with healthy subjects. In a cross-sectional study, we enrolled 80 patients on maintenance hemodialysis and measured the inflammatory and oxidative stress indicators. The plasma samples were assayed for five above mentioned variables using standard protocols. Two-hour post hemodialysis plasma samples were also assayed for TAC. Plasma levels of inflammation markers, IL-6 and hs-CRP, homocysteine were significantly increased in ESRD group versus control group. This increase was also found in TNF-α levels as compared to the controls, but the differences were not statistically significant. Also, the post dialysis samples had significantly lower levels of TAC as compared to predialysis ones.

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Introduction

Hyperhomocysteinemia is an independent cardiovascular risk factor in end-stage renal disease (ESRD) with a prevalence as high as 85 to 100% (2,3). Many dialysis patients are, therefore, at risk necessitating the development and testing of adequate treatment regimens, also plasma homocysteine concentration exhibits a strong association with indices of renal function. Hyperhomocysteinemia has been implicated in the high vascular event rate in patients with chronic renal failure. The precise pathophysiological explanation for the occurrence of hyperhomocysteinemia in renal failure is not yet elucidated a defective intrinsic renal metabolism of homocysteine seems unlikely. There are several indications that whole body homocysteine metabolism is altered in renal insufficiency. Several, but not all, prospective studies have linked hyperhomocysteinemia to adverse cardiovascular outcomes in renal failure patients. Treatment of hyperhomocysteinemia in renal insufficiency is based on folic acid containing regimens, but so far, none of the regimens has been shown to normalize plasma homocysteine concentration successfully. Intervention studies have not yet reported beneficial vascular effects of homocysteine-lowering treatment in dialysis patients. Moreover, oxidative stress is a consequence of an imbalance between reactive oxygen species (ROS) production and antioxidant capacity. After renal failure, antioxidant levels increase, possibly in response to increased generation of free radicals. As a result, increased lipid peroxidation may contribute to increased risks of atherosclerosis (4). The increase in total antioxidant capacity in patients with ESRD might be due to high serum uric acid. Plasma total antioxidant capacities decreased after HD in ESRD

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patients due to decreasing of uric acid concentration. This can occur as a result of either increased ROS generation impaired antioxidant system or a combination of both. In the presence of oxidative stress, the ROS attack will modify and denature functional and structural molecules leading to tissue injury and dysfunction factors such as exposure of blood to dialysis membranes, high risk of acute and chronic infections, and dietary limitations in the intake of the antioxidant vitamins make patients on dialysis susceptible to more oxidative stress. Tepel and colleagues have shown that in patients with ESRD, administration of the N-acetyl-cysteine a precursor for glutathione synthesis significantly reduced the incidence of cardiovascular events (5).

There are also some additional factors, Interleukin-6 (IL-6) and high-sensitivity C-reactive protein (hsCRP). Two inflammatory markers or mediators which are prone to an increased serum level in end-stage renal disease (ESRD), infections, cerebrovascular accidents, myocardial infarction, malignancies, and rheumatic diseases. TNF-alpha is a pro-inflammatory cytokine but mediates both other pro-inflammatory and anti-inflammatory cytokines. TNF-alpha has regulatory effect in plasminogen activator inhibitor (PAI-1) expression and secretion by vessel endothelial cells, the main fibrinolysis inhibitor, which its elevated levels are associated with an increased risk for vascular diseases (6). Tumor necrosis factor (TNF) in a variety of diseases has been reported, and tumor necrosis factor (TNF)-alpha causes hypertrophic as well as negative inotropic effects on cardiac myocytes. Circulating TNF-alpha concentrations are reported to be elevated in end-stage renal disease (ESRD) patients undergoing maintenance hemodialysis (HD) (6,7).

Hs-CRP was found to have direct pro-inflammatory effect on endothelial cells, and may be directly involved in the pathogenesis of vascular disease Chronic inflammation is a common feature of ESRD. It plays a key role in atherogenesis and development of atheroma. Inflammatory mechanisms with atherosclerotic plaque formation can be triggered, maintained and enhanced by multiple factors such as oxidized low-density lipoproteins, increased reactive oxygen species an activated macrophages that induce synthesis of neopterin. Therefore according to the important roles of above factors Current study was designed to elucidate the plasma concentrations related to the five predictive factors among Iranian patients with maintenance hemodialysis.

Materials and Methods

This was an observational-analytical study of five biomarkers generally associated with cardiovascular disease (CVD), a common outcome of end-stage renal disease (ESRD), which has been conducted in 2010. A total of 80 hemodialysis patients consecutively admitted to hemodialysis facilities of Hafte-tir and Pars hospitals of Tehran, capital of Iran, and equal numbers of age and sex-matched healthy subjects were enrolled in this study. Informed consents were obtained from all participants. Blood samples were taken using venipuncture (for controls; EDTA as anticoagulant) or access points (for hemodialysis patients) and soon after plasma samples were prepared and stored in -70°C (for Ferric Reducing Ability of Plasma, FRAP) or in 80°C (for 4 other assays) until used. One sample for each control and two samples (eight am and two hours after dialysis) for each case were taken. Determinations of plasma levels of five parameters, including homocysteine; total antioxidant capacity; CRP; IL-6 and TNF-α were carried out in duplicate experiments using standard protocols in the Department of Clinical Biochemistry of Tehran University of Medical Sciences.

Measurement

Total homocysteine concentration of plasma was measured with reversed-phase High Performance Liquid Chromatography (HPLC), an improved method of Vester and Rasmussen, using isocratic elution and fluorescence detection at 358 nm (excitation) and 515 nm (emission)(9). CRP levels of plasma were measured using High-sensitivity CRP detection kit based on enzyme-linked immunosorbent assay (hs-CRP kit, IBL, Germany, cat: EU59151). IL-6 and TNF-α cytokines of plasma were measured using enzyme-linked immunosorbent assay (Bender MedSystems, cat: BMS213/2 and cat: BMS223/4 respectively). Total antioxidant capacity of plasma samples were measured using Ferric Reducing Ability of Plasma (FRAP) method with colorimetery at 593 nm (9).

Mean values of duplicate experiments were calculated and considered to statistical comparisons. Kolmogorov-Smirnov method was used to test normality of collected data. Two independent samples *t*-test was used to compare means between patients and control subjects. Paired Samples t. test was used to compare mean values obtained from before and after hemodialysis samplings. Linear and multiple regression analysis were used to find correlations between different variables. Results are presented as means ±S.E.M. A *P*-

value <0.05 was considered to be statistically significant. All statistical analysis was done using SPSS 11.5 software.

Results

Eighty maintenance dialysis patients with mean dialysis vintage of 68 months (15 to 139 month) were enrolled in this study. Eighty healthy sex and age-matched subjects were also considered. Plasma samples were analyzed for five parameters and compared. Interrelations between five variables, sex, age and dialysis vintage, were explored. The following results was found: Plasma levels of inflammation markers, IL-6 and hs-CRP, homocysteine and TAC were significantly higher in ESRD patients compared to the healthy controls (3.79 ± 0.37 pg/mL, 9.52 ± 0.99 µg/mL, 21.54 ± 1.60 µg/L, 1188.13 ± 42.41 µmol/L against 2.3 ± 0.05 pg/mL, 3.18 ± 0.57 µg/mL, 10.2 ± 0.35 µg/L, 1066.15 ± 38.79 µmol/L, respectively). Post dialysis samples had significantly lower levels of TAC compared to pre-dialysis ones (1073.7 ± 19.56 against 1188.13 ± 42.41 µmol/L). TNF-α level was higher in patients compared to controls, but the differences were not statistically significant (12.32 ± 0.48 against 11.55 ± 0.33 pg/mL).

We found no association of sex or age with the antioxidants and acute-phase reactants levels in patients on maintenance hemodialysis. In our study, IL-6 and hs-CRP as, inflammation markers resulted in better prediction of inflammation than TNF-alpha. Plasma homocysteine levels of ESRD patients, in consistent with other studies, were higher than controls. Comparing pre and post hemodialysis levels of TAC revealed that a significant loss of plasma antioxidants are present during hemodialysis.

Means

Plasma levels of IL-6, hs-CRP, homocysteine and TAC were significantly higher in ESRD patients compared to the healthy controls (3.79 ± 0.37 pg/mL, 9.52 ± 0.99 µg/mL, 21.54 ± 1.60 µg/L, 1188.13 ± 42.41 µmol/L against 2.3 ± 0.05 pg/mL, 3.18 ± 0.57 mg/mL, 10.2 ± 0.35 µg/L, and 1066.15±38.79 µmol/L, respectively). Post dialysis samples had significantly lower levels of TAC compared to pre-dialysis ones (1073.7 ± 19.56 against 1188.13 ± 42.41 µmol/L). TNF-α levels were higher in patients compared to controls, but the differences were not statistically significant (12.32 ± 0.48 against 11.55 ± 0.33 pg/mL). Table 1 lists relevant demographic, clinical, and laboratory measures.

Correlations

Plasma homocysteine levels of healthy subjects were significantly higher in men than in women (8.42 ± 0.28 in females compared to 11.99 ± 0.29 in males, P<0.0001). The same differences were also seen among hemodialysis patients (15.93 ± 0.71 in females compared to 27.16 + 2.6 in males, P<0.0001). Plasma homocysteine levels tend to increase in aged patients or controls (R = +0.322, P = 0.043 for patients and R = +0.826, P < 0.0001) for healthy controls. Plasma hs-CRP levels showed strong correlations with age of healthy participants (R = +0.331, P = 0.037) but not in patients group, although the correlation was the strongest in total of 80 participants (R = +0.369, P<0.001). HD patients with upper dialysis vintages (in month) had lower TNF- levels (R = -0.327, P = 0.039). Other correlations were also seen between measured variables that the findings (R & P) have been presented in table 2.

Discussion

It has been proved that pro-inflammatory factors increase the risk of cardiovascular diseases and mortality especially in hemodialysis patients. Atherosclerotic cardiovascular disease is a major cause of mortality in patients with ESRD on maintenance HD. Chronic inflammation is a common feature of ESRD (10). Also, it plays a key role in atherogenesis and atheroma. Inflammatory mechanisms with athero- sclerotic plaque formation can be triggered, maintained and enhanced by multiple factors such as oxidized low-density lipoproteins, increased reactive oxygen species and activated macrophages that induce synthesis of neopterin. Hyperhomocysteinemia increases risk of coronary artery disease by increased thrombosis, adverse effects on endothelial function, promoting thickening of the intima and oxidative damage of low-density lipoprotein (12). The hyperhomocysteinemia was reported in 40% of patients with vascular disease (8) and lowered plasma homocysteine level would reduce the risk of CAD by16% (13).

Elevation in total plasma homocysteine (tHcy) is the first introduced predictor of CVD. Intracellular half-life of homocysteine is very short and efficiently is exported into the extracellular medium (10). Many factors may affect plasma concentrations of homocysteine. In addition current findings also are consistent with the reports, furthermore we found significantly higher homocysteine levels in ESRD patients (21.54 ±1.60

mol/L against 10.2 + 0.35 mol/L for controls) and the levels adequately correlated controls (Table 2). Besides in present research we found strong positive correlations between plasma homocysteine levels and hs-CRP or IL-6 in total population, but the correlations was lost among cases or controls probably due to sample size effect (Table 2). Elshamma and coworkers (2009) have reported enhanced oxidative stress and higher TAC or hs-CRP levels in HD patients. Oxidative stress (OS) is highly prevalent in patients with chronic renal failure who are on hemodialysis (HD) (1). It is suggested that HD imposes an additional OS to patients with chronic renal failure by the activation of granulocytes on dialyzer membranes, resulting in an imbalance between oxidants and antioxidants.

They also found positive correlations between these two markers (R = 0.52, $P < 0.08$). In this case TAC and hs-CRP levels among patients were found higher compared to controls (1188.13 ± 42.41 μmol/L TAC against 1066.15 + 38.79 μmol/L for controls, $P = 0.037$ and 9.52 + 0.99 μg/mL hs-CRP against 3.18 ± 0.57 mg/mL hs-CRP for controls, $P < 0.0001$), but in contrast to findings of Elshamma, there is a negative correlation in present data between hs-CRP and TAC only among healthy participants (R = -0.366, $P = 0.02$, Table 2). Nguyen-Khoa (2001) has reported significant reduction in plasma concentration of alpha-tocopherol and its negative correlation of dialysis treatment time with alpha-tocopherol (r = -0.49, $P < 0.02$) and ubiquinol (r = -0.40, $P < 0.05$). These findings indicate time dependent loss of plasma soluble antioxidants during HD and thus enhancement of oxidative stress in HD patients (11). We found a significant reduction in plasma antioxidant levels after HD (1188.13 mol/L for before HD against 1073.70 mol/L for after HD plasma samples, $P \leq 0.01$, Table 1). In patients with ESRD, IL-6 is not only a strong predictor of poor outcome; it is also independently associated with carotid atherosclerosis in dialysis patients. Clearly, regarding strong prognostic impact of IL-6 further studies are needed to elucidate the independent role of genetic predisposition, comorbidity, and renal dysfunction as causes of elevated IL-6 levels in ESRD patients. Proinflammatory cytokines such as TNF-α might also have atherogenic properties. TNF-α has been shown to regulate down. Apo E secretion, promote *in vitro* calcification of vascular cells and cause endothelial dysfunction (11,12).

Table 1. Demographic and laboratory characteristics of studied population

	Healthy	Cases	*P*-value
Age	40 + 0.92	50.33 + 1.05	-
Dialysis vintage (month)	-	69 + 4.39	-
Gender (%male)	%50	%50	-
TAC (μmol/L, before)	1066.15 + 38.79	1188.13 + 42.41*	*P*=0.037
TAC (μmol/L, after)	1066.15 + 38.79	1073.70 + 19.56	*P*=0.862
TAC (μmol/L, paired*)**		1073.70 + 9.56 (after)** 1188.13 + 42.41 (before)	*P*<0.01
Homocysteine (μmol/L)	10.2 + 0.35	21.54 + 1.60*	*P*<0.0001
hs-CRP (μg/mL)	3.18 + 0.57	9.52 + 0.99*	*P*<0.0001
IL-6 (pg/mL)	2.3 + 0.05	3.79 + 0.37*	*P*<0.0001
TNF-alpha (pg/mL)	11.55 + 0.33	12.32 + 0.48	*P*=0.228

* Differences are significant as compared to the healthy controls
**Differences are significant as compared to predialysis
***Paired: comparing TAC results before and after dialysis

It now appears that CRP is an active participant in pro-atherosclerotic phenomenon including local pro-inflammatory and thrombotic events. Studies in the general population indicate the usefulness of CRP in prognostication and in monitoring response to therapy. The clinical usefulness of CRP monitoring in chronic kidney disease (CKD) and especially in ESRD warrants more detailed study. Simmons (2005) has reported significant elevation in pro-inflammatory proteins IL-6, TNF-alpha, and CRP, as well as the oxidative stress markers in ESRD patients, compared with healthy control subjects (12). We also found higher IL-6 and hs-CRP levels among cases compared to control (see Table 1), and TNF-α levels in cases were also increased but the differences were not significant. Indeed, it has been previously noticed by many researchers that circulating levels of TNF-α may be influenced by a number of different factors and that circulating TNF-α levels may not reflect biologic activity at the tissue levels (13). Filiopoulos (2009) has reported significant increase in plasma levels of TAC, SOD and hs-CRP in HD and PD patients compared to healthy controls, and there were no

significant differences in TNF-alpha and IL-6 levels between the two groups (14). In this work, we realized a strong and positive correlation between IL-6 and CRP levels (Table 2), but the expected correlation between TNF- and IL-6 and CRP were not seen. Indeed, TNF-α is a cytokine that is produced in picogram amount and circulating levels of it does not reflect its true production levels, because its half-life is short and many cells have receptors to capture it from the circulation. There is strong evidence that serum concentration of IL-6 increases with age, but despite that present data does not support the fact, so we suggested it resulted potentially due to small size of the sample. Hutchinson (2000) reported the positive correlation of plasma CRP levels with age and its tendency to be higher in females (15). On the same result, there was a connection between age and plasma CRP levels (Table 2) but no significant correlations between CRP and gender in our population.

Table 2. cross correlations between variables

		Healthy		Patients		In a total population	
Correlation between		**R**	***P***	**R**	***P***	**R**	***P***
tHcy	**IL-6**	0.087	0.592	0.05	0.757	+0.292	0.009
tHcy	**TNF- α**	0.105	0.52	0.088	0.587	0.046	0.687
tHcy	**TAC**	0.099	0.544	0.012	0.941	0.161	0.153
tHcy	**Gender**	0.822	<0.0001	0.56	<0.0001	0.161	<0.0001
tHcy	**Age**	+0.826	<0.0001	+0.322	0.043	+0.612	<0.0001
tHcy	**HD vin**	-	-	0.177	0.276	-	-
IL-6	**Hs-CRP**	+0.326	0.04	+0.356	0.024	+0.473	<0.0001
IL-6	**TNF-□**	0.011	0.948	0.06	0.714	0.018	0.873
IL-6	**TAC**	-0.344	0.03	0.005	0.976	0.07	0.539
IL-6	**Gender**	0.07	0.666	0.153	0.347	0.104	0.359
IL-6	**Age**	0.091	0.576	0.149	0.358	0.199	0.077
IL-6	**HD vin**	-	-	0.209	0.196	-	-
hs-CRP	**TAC**	-0.366	0.02	0.012	0.94	0.016	0.89
hs-CRP	**Gender**	0.275	0.086	0.192	0.235	0.182	0.106
hs-CRP	**Age**	+0.331	0.037	0.117	0.474	+0.369	<0.001
hs-CRP	**HD vin**	-	-	0.072	0.657	-	-
TNF-α	**Hs-CRP**	0.069	0.674	0.059	0.716	0.027	0.814
TNF- α	**Age**	0.08	0.622	0.228	0.157	0.014	0.901
TNF- α	**Gender**	0.082	0.613	0.152	0.348	0.12	0.288
TNF- α	**HD vin**	-	-	-0.327	0.039	-	-
TAC	**TNF-α**	0.173	0.285	+0.334	0.035	0.164	0.146
TAC	**Age**	0.086	0.598	0.081	0.62	0.148	0.191
TAC	**Gender**	0.021	0.895	0.086	0.599	0.033	0.768
TAC	**HD vin**	-	-	0.026	0.874	-	-

R: regression coefficient & P: significance of the correlation
Cross-correlation calculated between two variables located in the 2 left columns
The signs - or + show negative or positive correlation/ hs-CRP: high sensitivity C Reactive Protein /tHcy: total plasma homocysteine / IL-6: Interleukin 6 / TNF-□: Tumor Necrosis Factor alpha / TAC: Total Antioxidant Capacity

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