Blood Enzymes and Oxidative Stress in Chronic Kidney Disease: A Cross Sectional Study

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Abstract. Background: Cardiovascular disease causes morbidity and mortality in chronic kidney disease. Increased formation of oxygen-derived radicals can accelerate the development of atherosclerosis. Several studies have shown an imbalance in antioxidant activity, resulting in high oxidative stress and lipid peroxidation, in subjects undergoing hemodialysis.

Methods: Eighty-nine subjects with chronic kidney disease and varying degrees of renal impairment, some of who were undergoing peritoneal or hemodialysis, were studied and compared with a group of healthy controls. F2-isoprostanes, selenium, and oxidized low density lipoprotein (LDL) concentrations were used as markers for in vivo oxidative stress; paraoxonase and glutathione peroxidase were used as markers of antioxidant activity. Results: We quantified serum F2-isoprostanes, paraoxonase, selenium, oxidized LDL, and RBC glutathione and compared the treatment regimens using post-hoc pair-wise comparisons and Tukey’s honest statistical difference. A significant increase in oxidative stress between the hemodialysis and control group was noted. The renal insufficiency and peritoneal dialysis groups showed increased oxidative stress and decreased antioxidant capacity, reaching statistical significance for some serum analytes. Selenium concentrations and oxidized LDL did not vary significantly between the hemodialysis and control groups. Conclusion: These data suggest that increased oxidative stress and decreased antioxidant activity are associated with declining renal function.

Key words: hemodialysis, chronic kidney disease, antioxidant, oxidative stress, atherosclerosis

List of Abbreviations: Low density lipoproteins (LDL); glutathione peroxidases (GSH-Px); paraoxonase (PON); high density lipoprotein (HDL); solid phase extraction (SPE); enzyme immunoassay (EIA); inductively coupled plasma-mass spectrometry (ICP-MS); enzyme-linked immunosorbent assay (ELISA); 3,3’5,5’-tetramethylbenzidine (TMB); erythrocytes (RBCs); analysis of variance (ANOVA); end stage renal disease (ESRD); selenium (Se)

Introduction

Complications of chronic kidney disease include cardiovascular disease, which is a major cause of morbidity and mortality in patients on hemodialysis. Patients on hemodialysis have abnormal lipid profiles which typically include elevated triglycerides, elevated very low density lipoproteins, elevated low density lipoproteins (LDL), and decreased high density lipoprotein (HDL) particles [1-4], which are common risk factors for cardiovascular disease. In conjunction with dyslipidemia, oxidative stress may increase the risk of cardiovascular disease in patients with chronic kidney disease [5;6]. Oxidative stress is an imbalance between pro-oxidant production and antioxidant defense mechanisms. Oxidative stress has been implicated in the pathology of several diseases, including cardiovascular disease and chronic kidney disease.

The role of antioxidant systems has been explored in subjects with chronic kidney disease. Studies have shown that subjects with chronic
kidney disease have impaired antioxidant systems as indicated by decreased glutathione peroxidase activity [7] and decreased copper and zinc superoxide dismutase [8]. Glutathione peroxidases (GSH-Px) are important selenium-containing enzymes that reduce hydrogen peroxides and protect against oxidative stress. GSH-Px are primarily synthesized in the kidneys [9;10] and are found in mammalian blood and tissues, with the highest concentrations found in the kidneys and liver. The activity of GSH-Px is affected by decreased blood selenium concentration and kidney disease. Subjects with chronic kidney disease tend to have decreased GSH-Px activity compared to those with normal kidney function [11]. Paraoxonase (PON), another marker of antioxidant activity, is an esterase enzyme associated with HDL and functions to protect LDL and HDL from oxidation. Both PON and GSH-Px activities are altered in subjects with renal failure [12-14].

Pro-oxidant systems have been evaluated in patients on hemodialysis. Oxidized LDL, a known mediator of atherogenesis, is elevated in patients with chronic kidney disease. Maggi et al. [15,16] showed that patients with uremia are more vulnerable to LDL oxidation than are normal individuals. F2-isoprostanes are another group of reliable markers of in vivo oxidative stress. F2-isoprostanes are prostaglandin-like substances that are produced when arachidonic acid undergoes lipid peroxidation [17]. They are present in all normal tissues and biological fluids; however, upon oxidative injury, F2-isoprostanes are significantly elevated [18]. They have been implicated as possible mediators of cardiovascular, neurological, renal, lung and liver diseases. These data suggest that oxidative stress may promote atherosclerosis in patients with end-stage renal disease.

The purpose of our study was to measure markers of pro-oxidant and antioxidant activity in subjects with various stages of chronic kidney disease (renal insufficiency, peritoneal dialysis, and hemodialysis) and to examine factors associated with increased serum F2-isoprostanes concentrations in subjects with renal disease. In addition to F2-isoprostanes, we examined two enzymes with antioxidant activities, PON and GSH-Px, which may play a role in modulating oxidative stress. We hypothesized that the serum F2-isoprostanes concentration will increase with decreasing renal function and that subjects undergoing hemodialysis would have the highest concentrations. Conversely, blood activities of PON and GSH-Px should be reduced in subjects with decreasing renal function, with the lowest activities occurring in subjects undergoing hemodialysis.

Materials and Methods
Eighty-nine subjects with chronic renal disease of varying degrees of renal impairment were studied and compared with a group of 30 healthy controls. The age range for the control subjects was 18 – 53 years old. Subjects in the control group consisted of adults with normal renal function. F2-isoprostanes, selenium, and oxidized LDL are used as markers for in vivo oxidative stress; PON and GSH-Px are used as markers of biologic antioxidant activity. The age range for the group with chronic renal disease was 20 – 84 years old. The subjects were sorted into renal insufficiency (not yet requiring dialysis), peritoneal dialysis, and hemodialysis groups to reflect the degree of renal impairment. There were 30 subjects in the group with renal insufficiency. The glomerular filtration rate ranged from 8 – 94 mL/min (median: 44.6 mL/min). In this group, 9 patients had diabetes, 17 had hypertension, none had cardiovascular disease, 1 had coronary artery disease, 1 had peripheral vascular disease, and 4 were on cholesterol lowering drugs (statins). There were 29 subjects in the group with peritoneal dialysis. The glomerular filtration rate ranged from 5 – 17 mL/min (median: 7.6 mL/min), 13 patients had diabetes, 26 had hypertension, none had cardiovascular disease, 8 had coronary artery disease, 3 had peripheral vascular disease, and 17 were on statins. There were 30 subjects in the group with hemodialysis. The glomerular filtration rate ranged from 5 – 13 mL/min (median: 7.5 mL/min). 14 patients had diabetes, 24 had hypertension, 5 had cardiovascular disease, 12 had coronary artery disease, 5 had peripheral vascular disease, and 6 were on statins.

After informed consent was obtained, samples were collected pre-hemodialysis and processed within 30 minutes. Serum samples were frozen on dry ice within 60 minutes of collection. Samples were stored at -70°C until analysis. No antioxidant cocktail was used in these samples. This study was approved by the Institutional Review Board of the University of Utah.

8-Isoprostane Enzyme Immunoassay
8-isoprostane concentrations were quantified using a competitive enzyme immunoassay (EIA) kit (Cayman
Chemical, Ann Arbor, MI, USA). The serum samples were prepared by combining 1.0 mL serum with 8.0 mL of de-ionized water and adjusting the pH to 3.0 with 1.0 N HCl. The samples were purified via solid phase extraction (SPE) using a C18 SPE cartridge. The column was first conditioned with 5.0 mL of methanol, followed by 5.0 mL of 1 mM HCl. The sample was loaded onto the column. The column was washed with 10 mL of 1 mM HCl, then with 10 mL of heptane. The 8-isoprostanes were eluted with 10.0 mL of ethyl acetate:heptane (1:1) and sodium sulfate was added to the eluate. The eluted 8-isoprostanes were purified through a second SPE silica column. The column was activated by pre-washing with 5.0 mL of methanol and 5.0 mL of ethyl acetate. The 8-isoprostanes were eluted with 5.0 mL of ethyl acetate:methanol (1:1) then evaporated under nitrogen gas. The dissolved residue was reconstituted in 500 µL of enzyme immunoassay (EIA) buffer for repeated immunoassay. The EIA was performed in duplicate on 50 µL of the reconstituted sample. Samples incubated for 18 hours at room temperature. After incubation, the assay was developed using Ellman’s Reagent. The reaction product was measured spectrophotometrically at 415 nm.

**Selenium**

Serum samples were prepared for analysis by combining 100 µL of serum, 100 µL of 1% nitric acid, and 4.8 mL of matrix matched diluent into a 10 mL trace element free polypropylene tube (royal blue top Vacutainer tube). Samples were analyzed for selenium by inductively coupled plasma-mass spectrometry (ICP-MS) using an ELAN 9000 DRC II ICP-MS instrument (Perkin-Elmer SCIEX). The instrument parameters were: forward power (coil) 1300 watts; argon plasma flow rate 15 L/min; argon auxiliary flow rate 1.1 L/min; nebulizer 0.8+/-0.1 L/min; mass calibration – m/z 82, 78; DRC parameters cell gas A (NH3) 0.55 L/min; lens voltage 5-10 volts; and autolens 3-13 volts.

**Oxidized LDL**

The oxidized LDL assay was performed using an oxidized LDL competitive enzyme-linked immunosorbent assay (ELISA), according to the package insert instructions (Merckodia Inc, Uppsala, Sweden). This kit uses a specific murine monoclonal antibody (4E6). The oxidized LDL in the sample competes with a fixed amount of oxidized LDL bound to the microtiter well for the binding of the biotin-labeled antibodies. The wells are washed to remove any unbound sample and HRP-conjugated streptavidin is used to detect the biotin-labeled antibody bound to the well. The wells are washed to remove excess HRP-conjugated streptavidin and the bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB).

**Paraoxonase**

The PON assay was performed using paraoxon as the substrate according to Furlong et al. [19]. Serum specimens were thawed and 760 µL of paraoxon assay buffer, which contained 2.63 M NaCl and 1.32 mM CaCl₂ in 132 mM Tris Buffer (pH 8.5), was added to 200 µL of thawed serum. The assay reaction was initiated by the addition of 200 µL of 6.0 mM freshly prepared paraoxon substrate solution at 37°C. The activity of PON was assayed by following the formation of p-nitrophenol by its absorbance at 405 nm. The absorbance was monitored continuously for 2 min. PON activity was also measured with phenyl acetate. Whole blood specimens were allowed to clot, after which the serum was separated by centrifugation lasting 15 min at 2000g. The serum aliquots were stored at -70°C until analysis. Serum specimens were thawed and diluted 1:40 with saline. The PON assay was performed using phenyl acetate as the substrate according to Dantoine et al. [12], with the following modifications: 800 µL of phenyl acetate substrate, containing 1.25 mM phenyl acetate, 0.9 mM CaCl₂ in 0.3 mM Tris-HCl buffer (pH 8.8), was added to 100 µL of diluted serum. The activity of paraoxonase was monitored by measuring the absorbance continuously at 270 nm for 1 min. PON activity is reported in U/L.

**GSH-Px Activity**

Venous blood specimens were collected in EDTA tubes. Plasma was removed from erythrocytes (RBCs). The RBCs were washed twice with cold saline and hemolyzed with ice-cold de-ionized water, then stored frozen until analysis. The total activity of erythrocyte GSH-Px was determined according to Andersen et al. [20], using tert-butyl hydroperoxide as the substrate. The absorbance was monitored at 340 nm for 300 sec. The final reaction volume was 250 µL and GSH-Px enzyme activity was reported in U/L. Serum GSH-Px was assayed by following the formation of 3,3',5,5'-tetramethylbenzidine (TMB).

**Statistical Methods**

Analysis of Variance (ANOVA) was used to compare the values of the serum concentrations of isoprostanes, GSH-Px, PON, selenium, and oxidized LDL across the varying degrees of renal impairment. Post-hoc pair-wise comparisons were calculated using Tukey’s honest statistical difference (HSD). The Spearman correlation coefficients were used to evaluate the inter-relationships between the analytes. All calculations were performed...
Table 1: Summary Statistics

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender (Males)</th>
<th>Age (Years)</th>
<th>F2-Isoprostanes (μg/L)</th>
<th>Oxidized LDL (μg/L 10⁻²⁻)</th>
<th>Paraaxonase Paraoxonase (U/L)</th>
<th>Phenyl Acetate (U/L)</th>
<th>Serum GSH-Px (μL)</th>
<th>RBC GSH-Px (μL)</th>
<th>Selenium (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>18-54</td>
<td>62.8 ± 16.6</td>
<td>6.2 ± 2.0</td>
<td>281.3 ± 154.5</td>
<td>115.1 ± 37.7</td>
<td>52.4 ± 18.2</td>
<td>10.6 ± 1.7</td>
<td>67.5 ± 6.2</td>
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<tr>
<td>Renal Insufficiency</td>
<td>18</td>
<td>21-72</td>
<td>91.7 ± 30.1</td>
<td>11.1 ± 4.1</td>
<td>137.0 ± 72.4</td>
<td>93.9 ± 31.9</td>
<td>34.1 ± 8.2</td>
<td>8.9 ± 2.8</td>
<td>63.7 ± 12.2</td>
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<tr>
<td>Peritoneal</td>
<td>18</td>
<td>22-78</td>
<td>108.3 ± 55.0</td>
<td>8.3 ± 4.3</td>
<td>192.9 ± 136.6</td>
<td>87.2 ± 20.5</td>
<td>28.3 ± 5.5</td>
<td>5.6 ± 2.1</td>
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<tr>
<td>Hmodidylacine</td>
<td>13</td>
<td>23-84</td>
<td>125.7 ± 40.5</td>
<td>7.4 ± 3.0</td>
<td>114.0 ± 58.4</td>
<td>74.9 ± 22.4</td>
<td>26.5 ± 6.1</td>
<td>9.6 ± 0.9</td>
<td>61.3 ± 15.2</td>
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The results for the seven analytes are represented as mean ± the standard deviation (SD).

Table 2: Spearman Correlation Coefficients and p-values

<table>
<thead>
<tr>
<th></th>
<th>Selenium</th>
<th>RBC GSH-Px</th>
<th>Serum GSH-Px</th>
<th>F₂⁻ Isoprostanes</th>
<th>Paraoxonase</th>
<th>Paraoxonase Phenyl Acetate</th>
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<tbody>
<tr>
<td>RBC GSH-Px</td>
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<td></td>
<td>p = 0.733</td>
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<td>Serum GSH-Px</td>
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<td>Paraoxonase Phenyl Acetate</td>
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<td>p = 0.0001</td>
<td>p = 0.0001</td>
<td>p = 0.0001</td>
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<tr>
<td>Oxidized LDL</td>
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<td></td>
<td>0.19503</td>
<td>0.0156</td>
<td>0.01594</td>
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<td></td>
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<td>p = 0.8838</td>
<td>p = 0.0789</td>
<td>p = 0.6533</td>
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Statistical significance = p < 0.05
Results

Serum concentrations of F2-isoprostanes, oxidized LDL, PON, GSH-Px, RBC GSH-Px and selenium were quantified. One group of controls and three groups of subjects with various degrees of renal impairment were compared using one-way analysis of variance (ANOVA). Post-hoc pair-wise comparisons were calculated using Tukey’s honest statistical difference (HSD). The means and standard deviations are listed in Table 1 for each analyte and subject group. There was a statistically significant increase (p < 0.05) in F2-isoprostanes in the three groups of subjects with chronic kidney disease compared to the control group (Figure 1A). Serum F2-isoprostane concentrations, a marker of in vivo oxidative stress, were elevated in all 3 groups with renal impairment; the largest increase was seen in subjects undergoing hemodialysis. According to the Spearman correlation, F2-isoprostanes had statistically significant negative correlations with RBC GSH-Px, serum GSH-Px, PON, and PON phenyl acetate (Table 2). Oxidized LDL, a marker of atherosclerosis, was found to be highest in subjects with renal insufficiency and the concentrations were statistically different from control subjects (Figure 1B). The peritoneal and hemodialysis groups had elevated oxidized LDL, but concentrations were not statistically different from the controls. Notably, the peritoneal and hemodialysis groups were statistically different from the renal insufficiency group. In addition, oxidized LDL had a statistically significant positive correlation with selenium (Table 2) but did not have statistically significant correlations with the other analytes.

PON, a marker of antioxidant activity, was evaluated using paraoxon and phenyl acetate substrates (Figures 2A and 2B). PON activity was decreased in all chronic kidney disease groups and was statistically significant from control for both substrates. The hemodialysis group had the lowest PON activity of the three groups. PON (paraoxon and phenyl acetate) also had statistically significant positive correlations with RBC and serum GSH-Px and a negative correlation with F2-isoprostanes (Table 2). Of note, PON paraoxon and PON phenyl acetate had a positive correlation with one another (Table 2). GSH-Px, measured in both serum and RBCs, was decreased in all chronic kidney disease groups and was statistically significantly decreased from the control group (Figures 3A and 3B). The greatest decrease in GSH-Px activity in both serum and RBCs was observed in subjects on hemodialysis. Serum and RBC GSH-Px had statistically significant positive correlations with PON and PON phenyl acetate and a negative correlation with F2-isoprostanes (Table 2). RBC and serum GSH-Px had a positive correlation with one another (Table 2). There was no statistically significant difference in selenium concentrations between subjects with kidney disease and subjects with normal kidney function (Table 1).
Oxidative stress is one of several factors that can advance the development of cardiovascular disease in patients receiving hemodialysis [6]. Cardiovascular disease is a major cause of morbidity and mortality in patients with chronic kidney disease. This study aimed to explore the role of antioxidant and pro-oxidant systems that contribute to cardiovascular disease in patients with kidney disease. Results from this study revealed that subjects on hemodialysis, with the greatest amount of renal impairment, had the highest amount of oxidative stress among all subject groups, as indicated by elevated serum marker F2-isoprostanes. Subjects with the greatest amount of renal impairment also had a corresponding significant reduction in antioxidant activity among all subject groups, as indicated by decreased PON and GSH-Px activity. These results demonstrate that pro-oxidant and antioxidant systems have an inverse relationship in subjects with chronic kidney disease. Furthermore, data from our study supports previous work which showed a reduction in antioxidant protection in subjects with uremia [12-14, 21].

Oxidized low density lipoproteins (oxidized LDL) are generated from an overproduction of reactive oxygen species and are known initiators of atherosclerosis [22, 23]. In 1994 Maggi et al. [15] reported that subjects with uremia have elevated oxidized LDL compared to subjects with normal kidney function. In addition, subjects with uremia also generated auto-antibodies against oxidized LDL [16], which supports previous findings that uremic subjects have a higher risk of cardiovascular disease. In 1996, Itabe et al. [24] also reported that hemodialysis patients have elevated plasma oxidized LDL concentrations, eight-fold higher than subjects with normal kidney function. These findings support the hypothesis that patients with chronic kidney disease have increased risk of cardiovascular disease due to the initiation and acceleration of atherosclerosis. In our study, oxidation was measured
in subjects with various stages of kidney disease. We found that oxidized LDL was slightly elevated in the peritoneal and hemodialysis groups, but was not statistically different from the control group. Surprisingly, subjects with renal insufficiency had higher concentrations of oxidized LDL than the other two kidney disease groups and were statistically different from control. Nevertheless, results from our study support previous findings that oxidized LDL is elevated in subjects with chronic kidney disease.

F2-isoprostanes are formed as a result of oxidative damage, such as acute ischemia, and are used as sensitive markers of in vivo lipid peroxidation [25] and oxidative stress in atherosclerosis [26]. Some studies suggest that F2-isoprostanes may be mediators of atherosclerotic disease [26,27]. Elevated F2-isoprostanes are also seen in the plasma of cigarette smokers [28] and patients with type II diabetes [29]. In 2001, Handelman et al. [30], measured F2-isoprostanes in patients with end stage renal disease (ESRD) and found that F2-isoprostanes were significantly elevated compared to subjects with normal kidney function. Ikizler et al. [31] supported this finding in 2002 and reported that F2-isoprostanes were elevated in chronic hemodialysis patients. Such data support the hypothesis that patients with ESRD have increased oxidative stress. Results from our study support previous findings. Serum F2-isoprostane concentrations were elevated in all 3 groups with renal impairment; the largest deviation from normal occurred in subjects on hemodialysis. In addition, data from our study also suggest that F2-isoprostanes may be a better marker for oxidative stress than oxidized LDL.

The role of antioxidant systems has been explored in patients with chronic kidney disease. Selenium (Se) is a trace element that is an essential structural component of RBC GSH-Px. GSH-Px functions as an antioxidant whose activity is regulated by the Se concentration in blood. Se deficiency is associated with decreased GSH-Px activity [32]. GSH-Px activity can be enhanced with Se supplementation in Se-deficient patients with normal kidney function [33]. The kidneys regulate the concentration of Se in the blood [34], and there are studies to support a parallel relationship between Se concentrations in blood and kidney function. For example, in 1995, Kallistratos et al. [35] reported that Se concentrations were decreased in whole blood and plasma as kidney disease progressed to ESRD. It was further noted that Se was also decreased in patients undergoing continuous ambulatory peritoneal dialysis [36]. On the contrary, it has been reported that Se concentrations do not differ between chronic kidney disease patients and subjects with normal kidney function [34,37]. Results from our study support the latter finding, namely that blood Se concentrations were not significantly different among normal control subjects and those with chronic kidney disease.

Decreased GSH-Px activity in blood is a common symptom in patients with chronic kidney disease. GSH-Px is one of several enzymes that function to reduce free radicals. This enzyme is primarily synthesized in the kidney [10]. There are two forms of GSH-Px that are found in whole blood: GSH-Px 1 found in red blood cells, and GSH-Px 3, found in plasma. Plasma GSH-Px activity is consistently decreased in patients with chronic kidney disease [38]; however, there has been a discrepancy in whether RBC GSH-Px activity is affected in the same manner. Several investigators have shown that RBC GSH-Px activity is significantly lower in patients with chronic kidney disease than in subjects with normal kidney function [14,38,39]. However, other investigators have shown that RBC-GSH Px activity was not significantly different from normal controls [40-42]. Results from our study demonstrated that both plasma and RBC GSH-Px were decreased in patients with various stages of chronic kidney disease.

PON is an esterase enzyme that functions to protect LDL and HDL from oxidation by metabolizing lipid peroxides which prevent macrophage foam cell formation [43]. PON activity is decreased in several pathological conditions that are associated with cardiovascular disease, such as myocardial infarction, diabetes, and hypercholesterolemia [44]. Studies have also shown that PON is decreased in patients with uremia, who have an increased risk of cardiovascular disease, when compared to subjects with normal kidney function [13,45]. In our study, PON was used as a marker of
antioxidant activity. Phenyl acetate and paraxoxon were substrates that were used to measure PON activity. Results from this study demonstrated that PON activity was significantly less in patients with various stages of chronic kidney disease than in normal controls. Our data support previous findings and suggest that the loss of PON activity may put patients with chronic kidney disease at a higher risk for oxidative stress and cardiovascular disease, despite the lack of correlation to oxidized LDL.

Cardiovascular disease is a major cause of morbidity and mortality in patients with chronic kidney disease. It has been suggested that increased formation of oxygen derived radicals accelerates the development of atherosclerosis. The purpose of our study was to measure markers of pro-oxidant (F2-isoprostanes and oxidized LDL) and antioxidant (PON, GSH-Px, Se) activity in patients with various stages of chronic kidney disease (renal insufficiency, peritoneal dialysis, and hemodialysis). Results from this study revealed that subjects on hemodialysis, with the greatest amount of renal impairment, had the highest amount of oxidative stress among all subject groups, as indicated by elevated serum marker F2-isoprostanes. Subjects with the greatest amount of renal impairment also had a corresponding significant reduction in antioxidant activity among all subject groups, as indicated by decreased PON and GSH-Px activity. These results demonstrate that pro-oxidant and antioxidant systems behave inversely in subjects with chronic kidney disease. To date, this is the first study to evaluate such markers in subjects in various stages of chronic kidney disease.

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References


