Total and Corrected Antioxidant Capacity in patients undergoing hemodialysis.

Niki Malliaraki¹, Dimitris Mpliamplias², Marilena Kampa², Andrew N. Margioris¹, Elias Castanas²

Departments of Clinical Chemistry (1), and Experimental Endocrinology (2), University of Crete, School of Medicine, and University Hospital, Heraklion, GR-71110, Greece

ADDRESS ALL CORRESPONDENCE TO:
Dr Elias Castanas
Laboratory of Experimental Endocrinology
University of Crete, School of Medicine
P.O. Box 1393, Heraklion
GR-71110, Greece
Tel: +30 810 394580, Fax: +30 810 394581
e-mail: castanas@med.uoc.gr
SUMMARY

Oxidative stress may play a critical role in the vascular disease of end stage renal failure and hemodialysis patients. Studies, analyzing discrete analytes and antioxidant substances, or the integrated total antioxidant activity of human plasma during hemodialysis, give contradictory results. Recently, we have introduced a new automated method for the determination of Total Antioxidant Capacity (TAC) of human plasma. We have serially measured TAC and corrected TAC (cTAC: after subtraction of the interactions due to endogenous uric acid, bilirubin and albumin) in 10 patients before the onset of the dialysis session, 10 min, 30 min, 1 h, 2 h and 3 h into the procedure and after completion of the session. Our results indicate that TAC decreases, reaching the lowest level at 2 h. However, corrected TAC increases with \( t_{1/2} \) of about 30 min. Subsequently, these measurements were performed in 65 patients undergoing dialysis with different filters (36 patients with ethylene vinyl copolymer filter –Eval-, 23 patients with two polysulfone filters –10 with F6 and 13 with PSN140-, and 6 patients with hemophan). Three specimens were collected (0, 30, 240 min). The results of this second group confirmed our initial results, while no significant difference was found using either filter. Our results are discussed relatively to possible mechanisms of modification of endogenous antioxidants, and the interaction of lipid- and water-soluble antioxidants.
INTRODUCTION

Hemodialysis represents a chronic stress status for its recipients [1-3]. It has been shown that although life salvaging, this procedure, by the application of a modified circulation dynamics including the forced passage of blood through a number of filters, activates endogenous inflammatory mechanisms and induces chronic release of molecules resulting in an increased production of reactive oxygen species [reviewed in 4, 5]. In addition, uric acid, an endogenous metabolite eliminated by hemodialysis, possesses significant antioxidant activity [6], while fluctuations in other endogenous antioxidant systems (plasma proteins, vitamins, etc) may lead to major variations of the internal redox state [1, 3, 6-8].

Circulation of oxidative molecules has been associated to increased protein, carbohydrate and lipoprotein oxidation and the generation of atherosclerosis [9, 10]. Indeed, accelerated development of atherogenesis and a number of vascular episodes characterize patients with chronic renal failure subjected to hemodialysis. In these patients oxidative stress relies on two major components: (1) The dialysis membrane and (2) the microbial contamination or pyrogen content of the dialysate. Contact with the dialyzer membrane is regarded as the main activating factor for priming the neutrophil oxidative burst. It is known that a dialysis session with cellulose dialysis membranes triggers a massive increase in the phagocyte basal production of ROS. The generation of intracellular ROS in both monocytes and polymorphonuclear cells during a dialysis session is closely related to the membrane biocompatibility. On the other hand, trace amounts of endotoxin (LPS) in the dialysate are potent triggers of ROS production via the activation of polymorphonuclear cells. LPS may activate blood monocyte/macrophage system through the dialyzer membrane contributing to IL-1, IL-6 and TNFalpha induction.

Cross-sectional studies of dialysis patients reveal that the traditional cardiovascular risk factors, including the presence of hypertension or hypercholesterolemia, have relatively low predictive power, while markers of inflammation and the protein-calories ratio, as reflected by the concentration of albumin, pre-albumin and antioxidants, correlate in a higher degree with cardiovascular mortality. The pathophysiology of the disease process that links uremia, inflammation and
malnutrition with increased cardiovascular complications is poorly understood. Nevertheless, some hypotheses have been advanced, linked to the presence of oxidant stress and its sequelae as a unifying concept of cardiovascular disease in uremia [11]. This hypothesis provides a framework to explain the link that activated phagocytes provide between oxidative stress and inflammation and the synergistic role that malnutrition contributes to the increased burden of cardiovascular disease in uremia.

In order to counteract vascular disease, a number of preventive strategies have been recently introduced, during and after hemodialysis. They include the administration of antioxidants, the use of new biocompatible filters (presumably less immunogenic), and the addition of vitamin, hormone or trace metals [1, 2, 12-22]. However, although it is generally accepted that oxidative stress may result from dialysis therapy, no direct evidence exists confirming this hypothesis. A number of reports, either measuring specific analytes or enzymes [8, 14, 21, 23-29], or estimating the total antioxidant activity of the plasma [1-3, 6, 7, 9, 25, 28, 30, 31] give contradictory and non-conclusive results.

Recently, we have introduced a new automated method for the estimation of the plasma total antioxidant capacity [32]. We have used this assay in order to evaluate changes of the antioxidant capacity of patients undergoing hemodialysis. To confirm our data, we have also normalized them per a number of analytes, directly affecting redox potential, thus introducing the concept of “corrected antioxidant capacity”. Our results indicate that, although total antioxidant capacity of hemodialyzed patients shows a decrease during the procedure, the corrected antioxidant capacity increases, indicating that counterbalancing mechanisms might occur in human plasma, counteracting the loss of urate and other antioxidant metabolites.
MATERIAL AND METHODS

Patients

Ten patients dialyzed with an ethylene vinyl alcohol copolymer (Eval) filter were analyzed. Seven samples were obtained from each patient, before the initiation of dialysis, 30 min, 1, 2 and 3 hours into the session and upon its completion. Sixty-five additional patients were examined (32 males [age range 15-91 years, mean 62.7] and 34 females [age range 26-88 years, mean 60.9]) and 56 volunteer blood donors on a normal diet (38 males and 18 females, age range 21-52 years). Polysulphone dialysis membranes were used on 23 patients (F6: 10 patients and PSN140: 13 patients), hemophane membranes on 6 (GFS 12 Plus) and ethylene vinyl alcohol copolymer resin filters (Eval 1.6 or 1.3) on 36. An informed consent was obtained from all participants in the study. Three samples (times 0, 30 and 240 min) were withdrawn from each patient, while a single sample was obtained from each blood donor. on K3-EDTA. Plasma was immediately separated by centrifugation (2000 g, 4°C), aliquoted and stored at –80°C until assayed.

Determination of TAC

Plasma total antioxidant capacity (TAC) was measured on an Olympus AU-600 chemistry analyzer using the TAC kit, described previously [32] (Medicon SA, Gerakas, Greece). Briefly, antioxidants in the sample inhibit the oxidation (bleaching) of crocin from ABAP [2,2-Azobis-(2-amidinopropane) dihydrochloride] to a degree that is proportional to their concentration. The assay was performed at 37°C in the following steps: 2 µl of sample, calibrator or control were mixed with 250 µl of crocin reagent (R1) and incubated for 160 s. Subsequently, 250 µl of ABAP (R2) were added and the decrease in absorbance at 450 nm was measured 26 s later. Values of TAC were expressed as mmol/l of Trolox equivalents.

Routine clinical chemistry

Plasma uric acid, albumin, total and direct bilirubin, were determined on an Olympus AU-600 chemistry analyzer using Olympus reagents provided by Medicon SA.
(Gerakas, Greece), as follows: uric acid OSR6136, albumin OSR6102, total bilirubin OSR6112. For the group of ten patients dialyzed with biocompatible membranes (Eval), a full blood count (white blood cells, polymorphonuclear cells, hemoglobin concentration and hematocrit value) accompanied all serial measurements.

Statistics

Statistical analysis of data was performed by the use of the SyStat v 10.0 program (SPSS Inc, Chicago, IL), and the Origin v 5.0 program (MicroCal, Northampton, MA).
RESULTS

Metabolite variation during dialysis.

Kinetics of a number of analytes during dialysis is presented in Figure 1. Uric acid concentrations decrease during dialysis, following an exponential decay curve with $t_{1/2}$ of 104 min. Albumin, on the other hand presents a gradual increase during dialysis, following an exponential growth curve, with $t_{1/2}$ of 101±12.8 min. It is interesting to note that this albumin increase correlates with the observed hemoconcentration of patients, as hematocrite counts follows exactly the same pattern, a result obtained also in previous studies [33]. In contrast, minor changes of bilirubin were found during the dialysis cycle. Cholesterol on the other hand, as well as HDL and LDL cholesterol present minor changes during the dialysis cycle.

Total and corrected plasma antioxidant capacity during hemodialysis

Figure 2 presents the variation of TAC during dialysis, in the ten hemodialyzed patients. As expected, due to the presence of a number of endogenous metabolites dotted with antioxidant activity (for example uric acid) initial TAC values of hemodialyzed patients are high, as compared to those of control individuals. During hemodialysis however, these elevated values decrease, according to an exponential decay model, with $t_{1/2}$ of 24.8 min. Thereafter, they remain constant during the whole time of hemodialysis.

Corrected TAC is also depicted in Figure 2. As stated in our previous work [32], this calculated parameter represents the fraction of circulating antioxidants, after the elimination of interference of endogenous metabolites. We have shown already that uric acid and bilirubin, and to a lesser degree albumin, are the major analytes interfering linearly with coefficients of 0.11, 0.11 and 0.01 mmol/L of TAC per mg/dL of each analyte respectively. As a result, we have calculated the corrected TAC values in the same patients. As shown in Figure 2, corrected TAC increases during the dialysis procedure, following a sigmoidal curve, with $t_{1/2}$ of 174 min. It is interesting to note that this value is slightly higher from the $t_{1/2}$ of uric acid decay (174±14.1 min, as compared to 101.2±12.8 min respectively).
Comparing TAC and corrected TAC values with those obtained in normal blood donors (depicted in Figure 2 as up and down triangles respectively), it is observed that total TAC values are significantly decreased ($t=3.75$, $p<0.001$) in hemodialyzed patients as compared to controls. In contrast, while initial corrected TAC values are significantly lower than those of controls ($t=2.97$, $p<0.01$), they reach normal values at the end of dialysis.

Effect of different dialysis filters on metabolites and TAC values

It has been suggested that hemodialysis-related oxidative burden relies greatly on the dialyzer membrane used. In this respect, as antioxidants might be consumed during a surge of oxidative molecules, TAC (as well as corrected TAC) might be decreased with the use of different membranes. We therefore attempted to investigate the effect of filters on TAC and corrected TAC values, in a number of 65 patients (33 males and 32 females). Thirty six patients were dialyzed using a biocompatible ethylene vinyl alcohol copolymer (Eval) filter. In 10 and 13 patients an F6 and PSN140 polysulfone filters respectively. Finally, 6 patients were dialyzed using a GFS12+ hemophane filter. These results are presented in Figure 3. No significant differences were found between the groups (Kruskal-Wallis test statistics with $p>0.05$ in any case), indicating that the filters used in the present study most probably do not modify significantly the redox state of patients, during the hemodialysis procedure.
DISCUSSION

The primary defence against oxidative stress in extracellular fluids results from a number of low molecular weight antioxidant molecules being either water- (ex. ascorbic acid) or lipid-soluble (ex. Vitamin E). These antioxidants are either generated during normal metabolism (ex. uric acid, bilirubin, albumin, thiols) or introduced in the body by the consumption of dietary products rich in antioxidants (olive oil, fruits and vegetables, tea, wine, etc) [34]. The sum of endogenous plus exogenous (food-derived) antioxidants represents the total antioxidant capacity of extracellular fluids. Changes of these antioxidants reflect their consumption during acute oxidative stress states. It should be noted that cooperation between different antioxidant pathways provides greater protection against attack by reactive oxygen or nitrogen radicals, compared to any single compound. Thus, the overall antioxidant capacity may give more relevant biological information compared to that obtained by the measurement of individual biomarkers, as it considers the cumulative effect of all antioxidants present in plasma and body fluids [35]. A hypothesis has recently been proposed, based on the redox potentials of exogenous and endogenous antioxidants. It postulates a cascade of reactions, in which following an oxidative stress, a lesser antioxidant is regenerated from a more potent one. Through this cascade, interactions between the hydrophobic and the hydrophilic phases of solutions could be established [36].

A variety of methods have been proposed for the assay of total antioxidant activity or capacity in serum or plasma [reviewed extensively and critically in 34, 35]. The fine distinction between antioxidant activity and antioxidant capacity should be elucidated here: Antioxidant activity corresponds to the rate constant of a single antioxidant against a given free radical; antioxidant capacity, on the other hand, is the number of moles of a given free radical scavenged by a test solution, independently of the capacity of any one antioxidant present in the mixture [35]. In the case of plasma, being a heterogeneous solution of diverse antioxidants, the antioxidant status is better reflected by antioxidant capacity rather than activity alone. This capacity is a combination of all redox chain antioxidants, including several analytes such as thiol bearing proteins, and uric acid. It thus appears that plasma antioxidant capacity is rather a concept than a simple analytical determination. Indeed, an increase of
antioxidant capacity of plasma indicates absorption of antioxidants and improved in vivo antioxidant status [37], or the result of the activation of an adaptation mechanism to oxidative stress. It should be noted that, due to the contribution of diverse metabolites to the antioxidant capacity of human plasma, its increase may not necessarily be a desirable condition. Indeed, in some cases, such as renal failure (uric acid), icteric status (bilirubin), hepatic damage (hypoalbuminemia) the increase or decrease of several metabolites modify plasma antioxidant capacity in a deleterious direction, a situation returning to normal values after correction of the underlying disease [38]. Recently, we have introduced a new automated method for the assay of the plasma antioxidant capacity, based on the bleaching of crocin. This method (the TAC assay) gives an estimation of the integrated plasma antioxidant capacity. Furthermore, we also determine the interference of a number of endogenous analytes, such as uric acid, and bilirubin, which have been found to produce a major interference of TAC, while albumin results in a smaller interference [32]. The substraction of these interferences in TAC assay resulted in the calculation of corrected TAC, representing the amount of antioxidant capacity due to the action of (mainly) exogenous antioxidants.

In the present work, we have assayed simultaneously TAC and the concentrations of these analytes during a single episode of hemodialysis. As expected, major modifications of these parameters were observed during dialysis. Namely, uric acid is rapidly eliminated, with t_{1/2} of 101 min (Figure 1), while albumin plasma concentration is increased (t_{1/2} 21 min) probably due to the hemoconcentration (ah hematocrit follows exactly the same patern) during dialysis. Bilirubin, on the other hand, follows a biphasic pattern with an initial decrease (possibly due to elimination) followed by a slight increase due to hemoconcentration. A pattern similar to that of direct bilirubin was observed for the total TAC measurements. Indeed, results presented in Figure 2 show that the plasma antioxidant capacity of patients is higher before than during or after a session of dialysis. This can be due to the elimination of a number of metabolites, such as uric acid and bilirubin (compare Figures 1 and 2). In this respect, our results are similar to those presented in previous reports, in which a comparable decrease of plasma antioxidant capacity was observed during renal dialysis [2, 3, 8-10, 14, 25, 31, 39, 40]. Total TAC activity was found to increase later.
in hemodialysis, most probably due to either hemoconcentration [30], adaptation [41], or to a possible exchange of antioxidants between the lipid and aqueous phases [36].

Calculation of the corrected TAC (in which the interference of a number of endogenous metabolites has been subtracted) appears to provide a better estimate of the actual antioxidant activity of the organism, especially in cases such as renal dialysis, in which major fluctuations of endogenous metabolites occur. Indeed, as shown in Figure 2, the curve of corrected TAC is different from that of TAC. Specifically, a gradual increase of plasma antioxidant capacity is observed, with $t_{1/2}$ of about 30 min. Various explanations for these results could be proposed: (1) Water elimination during dialysis occurs in increased concentration of endogenous antioxidant substances [30]. (2) Elimination of uric acid modifies the equilibrium between oxidized and reduced state of endogenous and exogenous antioxidants [41, 42]. (3) It has been recently proposed that elimination of water-soluble metabolic antioxidants (bilirubin, uric acid) modifies the equilibrium of lipid- and water-soluble antioxidants [36].

Redox state in uremic patients undergoing dialysis is rather confusing. Several reports provide possible pathophysiological explanations of the observed changes in redox state and antioxidant status. The can be summarized in the following:

1. **Inflammation and oxidative stress**: 30-50% of predialysis, hemodialysis and peritoneal dialysis patients have serologic evidence of an accelerated inflammatory response [43]. Phagocytes and cytokines may contribute to increased production of ROS [44-47].

2. **Malnutrition and oxidative stress**: Several cross-sectional studies indicate that hypoalbuminemia correlates with cardiovascular mortality in ESRD patients [48, 49]. It should be noted that, in plasma, the most important antioxidant is provided by thiol groups, which are largely located on the albumin molecule [50, 51]. In addition to possessing active antioxidant moieties, albumin has been shown to function as a “suicide scavenger” preventing oxidative injury to lipoproteins and the vascular wall [52]. It appears that patients with malnutrition and low plasma albumin concentration have significantly less
plasma antioxidant capacity, as compared to normals, due to diminished availability of thiol groups.

3. Retained uremic solutes may become substrates for oxidative injury: Several lines of evidence indicate that further oxidative modification of retained solutes in the uremic milieu (ex. β2microglobulin, homocysteine, cysteine) may potentiate their pathogenicity [53-55].

4. Dialytic therapy, which acts to reduce the concentration of oxidized substrates, improves the redox balance [11, 56]. However, processes related to repetitive extracorporeal dialytic therapies (prolonged use of catheters for vascular access, use of bioincompatible dialysis membranes) may produce more inflammatory and oxidative stimuli (via complement and leukocyte activation), thus contributing to a pro-atherogenic state [33, 57]. Our data, presented in Figure 3 show no major changes in both analytes and TAC as a result of filters used.

In conclusion, our data suggest that although during hemodialysis several factors contribute to the generation of oxidative radicals, the organism has the capacity to counteract the flood of oxidative substances. Indeed, the corrected TAC activity suggests that antioxidant supplementation may not be necessary in hemodialysis. Oxidation and peroxidation reactions of renal failure patients must be reevaluated under this point of view, taking into account the auto-oxidation of excess antioxidants, as was recently reported for vitamin C and tocoferols [58-61], rather than a decrease of the plasma antioxidant capacity.
REFERENCES


LEGENDS TO FIGURES

Figure 1
Variation of uric acid, albumin, hematocrit and bilirubin during dialysis
Data obtained from 10 dialysis patients. Mean±SEM are depicted.

Figure 2
Variations of TAC and corrected TAC during dialysis
Up and down arrows depict values obtained in normal blood donors. Mean±SEM of 10 patients (curves) and 56 blood donors (up and down triangles).

Figure 3
Effect of different filters used in dialysis on the concentration of different analytes and TAC levels
Parameter variation in 65 patients under dialysis. 23 patients were dialyzed with polysulphone dialysis membranes (F6: 10 patients and PSN140: 13 patients), 6 patients with hemophane membranes (GFS 12 Plus) and in 36 patients ethylene vinyl alcohol copolymer resin filters (Eval 1.6 or 1.3) were used. Mean±SEM of vaues.
Figure 3