Critical evaluation of plasma and LDL oxidant-trapping potential in hemodialysis patients

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Background. We investigated whether the total peroxyl radical-trapping antioxidant potential (TRAP) assay, which has recently been proposed as a gauge of oxidative stress, could serve to evaluate plasma and low density lipoprotein (LDL) antioxidant state in hemodialysis (HD) patients.

Methods. TRAP was determined by the lag time of the chemiluminescence reaction induced by azo-initiator-catalyzed linoleic acid peroxidation in the plasma and corresponding LDL preparations of 23 HD patients and 22 healthy subjects. Antioxidant systems, including glutathione peroxidase (GSH-Px), ascorbate, vitamin E, and uric acid, oxidative stress markers including malondialdehyde (MDA), carbonyls, and advanced oxidation protein products (AOPP), and lipids, including cholesterol and triglycerides, were also determined in the plasma.

Results. Both plasma and LDL-TRAP were significantly increased in HD patients despite decreased GSH-Px and ascorbate and increased MDA, carbonyl, and AOPP plasma levels. Plasma TRAP values were closely related to both uric acid and AOPP levels, and LDL-TRAP values were related to triglycerides and AOPP levels. In vitro studies showed that: (a) plasma TRAP of control plasma increased regularly with supplementation of uric acid, although not reaching that of HD plasma with similar uric acid levels; (b) the addition of human serum albumin-AOPP also regularly increased control plasma TRAP, but was close to that of HD plasma with similar AOPP levels; and (c) LDL-TRAP was increased following LDL enrichment with triglycerides.

Conclusion. Our study demonstrates that TRAP is not a relevant parameter for evaluating plasma or LDL antioxidant capacity in HD patients, due to the high plasma levels of uric acid, triglycerides and AOPP, which by themselves do not exert efficient antioxidant activity in vivo, but in vitro are able to scavenge the peroxyl radicals involved in the TRAP assay.

Key words: total peroxyl radical-trapping antioxidant potential, antioxidant, low density lipoprotein, carbonyls, malondialdehyde.

Chronic renal failure (CRF) is associated with an increased risk of atherosclerotic cardiovascular disease [1, 2], which represents the major cause of mortality in patients on long-term hemodialysis (HD) [3–5]. Oxidative stress, defined as a rupture in the pro-oxidant–antioxidant balance in favor of the former, largely participates in the pathogenesis of atherosclerosis [4, 5]. Interestingly, oxidative stress has recently been incriminated in the progression of renal diseases [6, 7], and culminates in dialysis patients who combine a massive generation of reactive oxygen species at each dialysis session [8–12] with a chronic deficiency in the major antioxidant systems [13–16]. Increased serum concentrations of lipid peroxidation by-products such as malondialdehyde (MDA) have been reported in HD patients, although with conflicting results [17, 18]. More recently, we reported the presence of advanced oxidation protein products (AOPPs) as novel markers of oxidative stress and proinflammatory mediators in both dialyzed [19] and nondialyzed CRF patients [20]. Interestingly, Miyata et al suggested that oxidative stress could also participate in the formation of advanced glycation end-products (AGEs) [21, 22] in HD patients, and we reported the accumulation of the AGE pentosidine with the progression of CRF [23].

The total peroxyl radical-trapping antioxidant potential (TRAP), measured by the ability of a biological fluid to resist 2,2′-azobis [2-amidinopropane] hydrochloride (ABAP)-induced linoleic acid peroxidation, has recently been proposed as a useful tool for determining the global antioxidant capacity of plasma in patients with oxidative stress-associated diseases such as diabetes [24], sickle cell disease [25], schizophrenia [26], and cystic fibrosis [27]. Jackson et al have reported an increased plasma TRAP in HD patients and related this finding to the presence of high circulating levels of uric acid, which because of the profound ascorbate deficiency in these patients, has no efficient antioxidant activity in vivo [28].

The generation of oxidized low-density lipoprotein...
(ox-LDL) is a common feature of oxidative stress, and high levels of ox-LDL have been reported in HD patients [29, 30]. However, by their triglyceride content and their capacity to retain liposoluble antioxidants such as vitamin E, LDLs also contribute to the antioxidant status [31]. LDL-TRAP has been studied in normal subjects [32] but has not yet been investigated in HD patients.

This study was aimed at evaluating whether or not the TRAP assay could be used to quantitate the redox state of both plasma and LDL of HD patients assessed by circulating plasma levels of antioxidants including glutathione peroxidase (GSH-Px), ascorbate, vitamin E, and uric acid and oxidative stress markers, including MDA, carbonyls, and AOPP.

**METHODS**

**Patients**

Twenty-three nonsmoking HD patients (11 males and 12 females; mean age ± SD, 63 ± 15 years) entered this study after giving informed consent. Patients with chronic hepatitis, hematological and inflammatory disorders, cancer, and immunosuppressive therapy were excluded. End-stage renal disease was secondary to interstitial nephropathy (7 patients), polycystic kidney disease (4 patients), glomerulonephritis (3 patients), diabetic nephropathy (2 patients), nephroangiosclerosis (1 patient), or unknown origin (6 patients). All patients were dialedyzed for more than three months for 210 to 270 minutes three times per week with diacetate or triacetate membrane filters using bicarbonate dialysate. All patients had a serum intact parathyroid hormone level below 250 pg/ml.

Twenty-two nonsmoking healthy subjects (9 males and 13 females, aged 41 ± 13 years) were recruited among the laboratory staff and served as controls.

**Materials**

If not otherwise indicated, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**Blood collection, plasma and low-density lipoprotein isolation**

Venous blood (10 ml) from HD patients and controls was collected in standard tubes containing 5 mM ethylenediaminetetraacetic acid (EDTA). In HD patients, blood was drawn from the arteriovenous fistula just before the dialysis session. Following centrifugation (600 g × 10 min, 4°C), plasma was stored at −80°C. LDLs from 10 HD patients and 10 control subjects were isolated by precipitation of 1 ml of plasma with 7 ml of 64 mM citrate buffer, pH 5.05, containing 50,000 IU/liter sodium heparinate [33]. After centrifugation, the pellet was resuspended in 1 ml 100 mM phosphate-buffered saline (PBS), pH 7.4, containing 0.15 M sodium chloride.

**Total peroxyl radical-trapping antioxidant potential assay**

Plasma and LDL-TRAP were measured by the delay in the luminol-amplified chemiluminescence (CL) production induced by linoleic acid peroxidation initiated by ABAP [32]. Briefly, the generation of peroxyl radicals was induced by the injection of 100 µl of 250 mM ABAP (Polysciences Inc., Warrington, PA, USA) in the luminometer (AutoLumat LB 953; Berthold, Wildbad, Germany) cuvette containing the reaction mixture, that is, 20 µl of 120 mM linoleic acid, 50 µl of 3 mM luminol (5-amino-2,3-dihydro-1,4-phtalazinedione), and 30 µl of plasma in 470 µl of PBS or 100 µl of LDL in 400 µl of PBS. The CL reaction proceeds through the sequence of initiation, propagation, and termination phases and is characterized by its lag time (t lag), rate, and peak (Fig. 1). The CL t lag is lengthened by the presence of antioxidants such as Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid; Aldrich, Milwaukee, WI, USA), a water-soluble tocopherol that is used as a standard for quantitating TRAP measurement [34]. Assuming that each Trolox® molecule neutralizes two peroxyl radicals, TRAP is expressed as follows:

$$\text{TRAP (µmol/liter)} = \frac{[\text{t lag}_{\text{Sample}}/\text{t lag}_{\text{Trolox}}]}{8} \times \text{Trolox (µmol/liter)} \times 2$$
**Lipid and protein oxidative stress markers**

Plasma levels of MDA were measured by a highly sensitive, synchronous fluorescence technique [35]. Briefly, 100 μl of plasma were mixed to 750 μl of thiobarbituric acid reagent (Sobioda, Montbonnot, France) in acidic condition and boiled for one hour. After extraction with 3 ml of N-butanol, the spectrum of the organic layer was recorded on a spectrophotometer (Shimadzu RF540, Roucaire, Courtaboeuf, France) between 500 and 600 nm keeping a constant interval, that is, Δλ of 14 nm between excitation and emission wavelengths. The MDA concentration was calculated by the fluorescence peak intensity at 553 nm using tetramethoxypropane, which produces MDA, as the standard.

Plasma levels of carbonyls were measured by a spectrophotometric procedure using 2,4-dinitrophenylhydrazine (DNPH) [36]. Briefly, plasma was treated with 10 mM DNPH in 2.5 mM HCl. After incubation for one hour at room temperature in the dark, deproteinization with 20% (wt/vol) trichloracetic acid was realized at 4°C. The pellet was washed three times in ethanol/ethyl acetate (1:1, vol/vol) and then solubilized at 37°C for 30 minutes in 6 M guanidine hydrochloride, pH 2.3. Carbonyls were measured using molar absorption coefficient ε of 22,000 m⁻¹cm⁻¹ at 370 nm and expressed in nmol/mg of protein measured at 280 nm by referring to bovine serum albumin.

Plasma levels of AOPP were measured as previously described in our laboratory [19] by a spectrophotometric assay using a microplate reader MR5000 (Dynatech, Paris, France) at 340 nm. Briefly, 200 μl of a fivefold dilution of plasma in 10 mM PBS were placed in a 96-well plate (Nunc-immuno plate; Nunc, Roskilde Denmark) and mixed with 20 μl acetic acid. AOPP concentrations were expressed in μmol/liter by referring to standard wells, containing 200 μl of chloramine-T solution (0 to 100 μM), 10 μl of 1.16 M potassium iodide, and 20 μl of acetic acid.

**Antioxidant systems in plasma**

Seleno-dependent GSH-Px activity was measured in plasma using tert-butyl hydroperoxide as described previously [6]. Briefly, the reaction mixture consisting of 1 mM reduced glutathione, 1 mM tert-butyl hydroperoxide, 230 IU/liter of yeast glutathione reductase, 0.143 mM nicotinamide adenine dinucleotide phosphate (NADPH), and 1.5 mM potassium cyanide in 100 mM PBS. The assay kinetics were calculated using molar absorption coefficient ε for NADPH of 6.22 × 10⁻³ M⁻¹ cm⁻¹. GSH-Px activity was expressed in millimoles of NADPH oxidized per milliliter (IU/liter).

Plasma levels of vitamin C were measured by an automated enzyme-linked ferric-tripryridyltriazine spectrophotometric assay (Cobas Fara, Roche, Meylan, France) specifically determining ascorbate, the reduced form of vitamin C using ascorbate oxidase [37]. Briefly, two aliquots of 100 μl of plasma were prepared, one with 40 μl of 4 UI/ml ascorbate oxidase and the other with 40 μl of PBS. The reaction mixture consisted of 10 μl of sample with 30 μl of PBS and 300 μl of reagent prepared with 10 ml of 300 mM acetate buffer, pH 3.6, 1 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 0.04 N HCl, and 1 ml of 20 mM ferric chloride. The reduction of Fe³⁺-TPTZ in the Fe²⁺-TPTZ blue form was measured at 593 nm after an incubation of 45 seconds at 37°C. The ascorbate plasma level was calculated by the difference between absorption of sample with and without ascorbate oxidase. Ascorbate (0 to 250 μM) (Merck, Darmstadt, Germany) was used as the standard.

Plasma levels of vitamin E were determined by a reverse high-performance liquid chromatography (HPLC) procedure as described in [38], after deproteinization of 200 μl of plasma with 200 μl of anhydrous HPLC-grade ethanol. Briefly, after vitamin E extraction with 400 μl of HPLC-grade heptane, the organic layer was evaporated and under a nitrogen stream and the dry extract was redisolved in 400 μl of HPLC-grade methanol. One hundred microliters of the sample were injected in a 250 × 4.6 mm, 5 μm ODS-C18 column (Satisfaction RP18AB; Cluzeau, Ste Foy La Grande, France), and eluted with HPLC-grade methanol at a flow rate of 2 ml/min. Detection was by ultraviolet absorbance at 292 nm. Internal and external standards consisted in α-tocopherol and α-tocopherol acetate, respectively.

Plasma levels of uric acid were determined using a Hitachi 917 analyzer (Boehringer Mannheim, Meylan, France) with commercially available kits.

**Lipid determinations**

Cholesterol and triglycerides were determined in the plasma and/or LDL preparations (for triglycerides) using a Hitachi 917 analyzer.

**Advanced oxidation protein products**

*in vitro preparation*

Human serum albumin (HSA; fraction V; CalBiochem, Mendon, France) was oxidized with hypochlorous acid (HOCl) as described by Witko-Sarsat et al [19]. Briefly, HSA (100 mg/ml) was incubated at room temperature for 15 minutes with 100 mM HOCI (HOCl:protein molar ratio of 60:1) and then dialyzed overnight against 10 mM PBS, pH 7.4.

**Statistical analysis**

Data were expressed as means ± sd. Statistical analysis was performed using the Mann–Whitney U-test for nonparametric data. Simple regression analysis and Spearman’s rank correlation coefficients were calculated to
assess the relationship between the various parameters. The threshold of statistical significance was $P < 0.05$.

RESULTS

Plasma and LDL-TRAP in hemodialysis patients and control subjects

Plasma from HD patients significantly increased lag time and reduced both the rate and peak of the CL reaction as compared with control plasma (Table 1). LDL preparations from HD patients also significantly increased CL lag time but had no effect on the CL rate or peak. Therefore TRAP, which only accounts for CL lag time, was significantly higher in HD patients than in controls and for both plasma ($P < 0.001$) and LDL ($P < 0.05$; Table 1). Moreover, a positive correlation was observed between plasma TRAP and LDL-TRAP values ($r = 0.54$, $P < 0.05$).

Because previous studies have reported profound changes in antioxidant systems with aging [39, 40], we compared plasma TRAP values in HD patients aged less than 50 years ($N = 7$) or $\geq 50$ years ($N = 16$) to those in healthy controls aged less than 50 years ($N = 18$). Plasma TRAP values (mean $\pm$ sd) were similar in HD patients $< 50$ years or $\geq 50$ years (1364 $\pm$ 227 and 1290 $\pm$ 264 $\mu$mol/liter, respectively) and were significantly higher in HD patients $< 50$ years than in controls $< 50$ years (1364 $\pm$ 227 vs. 775 $\pm$ 161 $\mu$mol/liter, $P < 0.01$).

Relationship between TRAP and plasma levels of antioxidant systems

Plasma levels of GSH-Px and ascorbate were significantly decreased in HD patients as compared with controls (Table 2). In contrast, plasma levels of vitamin E were similar in HD patients and in control subjects.

Plasma TRAP values were inversely correlated with GSH-Px ($r = -0.59$, $P < 0.01$) and ascorbate ($r = -0.54$, $P < 0.01$) plasma levels. In contrast, LDL-TRAP values were not correlated with these antioxidants but were positively correlated with plasma levels of vitamin E ($r = 0.62$, $P < 0.01$).

In contrast to other antioxidants, uric acid was significantly higher in the plasma of HD patients than of control subjects, and its level was positively correlated with plasma TRAP ($r = 0.70$, $P < 0.0001$) but not with LDL-TRAP values.

Relationship between TRAP and plasma levels of lipid and protein oxidation products

Malondialdehyde, carbonyl, and AOPP concentrations were significantly increased in the plasma of HD patients as compared with controls (Table 2).

Plasma TRAP values were positively correlated with plasma levels of carboxyls ($r = 0.42$, $P < 0.02$) and AOPP ($r = 0.59$, $P < 0.0001$) but not MDA. In contrast, LDL-TRAP values were correlated with both MDA ($r = 0.60$, $P < 0.01$) and AOPP ($r = 0.60$, $P < 0.01$) but not with carbonyl plasma levels.

Relationship between TRAP and plasma lipid levels

Whereas plasma cholesterol levels were similar in HD patients and control subjects, plasma triglyceride levels were significantly higher in the former (Table 2).

Although no correlation was found between plasma TRAP and triglyceride levels, a positive correlation was observed between LDL-TRAP values and plasma triglyceride levels ($r = 0.64$, $P < 0.01$).

In vitro effect of compounds correlating with the TRAP in vivo

The positive correlations observed between, on the one hand, plasma TRAP and uric acid or AOPP plasma levels and, on the other hand, LDL-TRAP and triglycer-
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Recently, TRAP has been proposed as a relevant parameter to determine the total antioxidant capacity of plasma from patients with oxidative stress-associated diseases [24–27]. In contrast, the antioxidant potential of LDL has not yet been investigated, although it could be of great interest in the evaluation of the role of ox-LDL in the atherosclerosis process.

This study aimed at determining the potential clinical relevance of the TRAP parameter to gauge the redox state of HD patients showed that in fact both plasma and LDL-TRAP were significantly increased in these patients as compared with controls (Table 1). This was found despite high circulating levels of oxidative stress markers, including MDA, carbonyls, and AOPP, and a profound deficiency of major antioxidants, including GSH-Px and ascorbate but not vitamin E (Table 2), the activity of which, however, is known to be consistently impaired when the ascorbate level is reduced [41]. The inverse relationship found between plasma TRAP and plasma levels of GSH-Px or ascorbate further demonstrated that the capacity to scavenge peroxyl radicals in vitro did not reflect the activity of the major plasma antioxidant systems. Rather, it suggested the presence of other substances in the plasma of uremic patients that are not necessarily efficient chain-breaking antioxidants in vivo, but are able to exert a peroxyl radical-scavenging effect in vitro. Among these, uric acid is a good candidate. Uric acid is usually an efficient antioxidant in vitro. However, this is less the case in vivo in the presence of decreased ascorbate levels [42]. In addition, it has been shown that uric acid is not efficient against oxidant-mediated protein damage [43].

Interestingly, Jackson et al also found that plasma TRAP was higher in HD patients than in controls and that it was significantly decreased by the end of a single dialysis session [28]. They attributed these observations to uric acid, which is present at high plasma levels before the dialysis session and dramatically decreases at the end of the session. Our in vitro study showed that the addition of uric acid to normal plasma increased its TRAP in a dose-dependent manner and thus further supported the major influence of uric acid on TRAP assay. However, our observation that the control plasma TRAP obtained after uric acid supplementation was still lower than that of an HD plasma with a similar uric acid level (Table 3) strongly suggested that other uremia-related substances contributed to the increase in plasma TRAP of HD patients.

Among these, AOPP, which we have recently proposed as novel uremic toxins and molecular basis of oxidative stress [44, 45], should be considered despite the fact that they do not exert proper antioxidant activity. Indeed, Hawkins and Davies have previously reported that oxidized proteins become more avid than native proteins for trapping radicals [43]. It is thus tempting to propose...
that AOPPs, which are present at high concentration in the plasma of HD patients, could exert a scavenging effect \textit{in vitro} on the ABAP-catalyzed peroxyl radicals involved in the assay. Our results showing that (a) \textit{in vivo} plasma TRAP values were correlated with AOPP levels but not with MDA levels, and (b) \textit{in vitro} supplementation of normal plasma with HSA-AOPP preparations significantly increased TRAP values (Table 3) support this hypothesis.

Our finding that LDL-TRAP of HD patients was, like plasma, significantly increased allows the following comments. The presence of ox-LDL, as a consequence of oxidative stress, has been well documented in HD patients [29]. The oxidation process may affect lipid and/or protein moieties of LDL [46, 47], and interestingly, LDL-TRAP values were closely related with both MDA and AOPP plasma levels. Because dialysis triggers a massive generation of chlorinated oxidants [8, 9, 11, 12], which represent the major source of oxidized proteins [48, 49], it is tempting to propose that, as discussed earlier in this article for plasma, the ox-LDL protein moiety (LDL-AOPP) may delay the CL reaction by scavenging the peroxyl radicals generated in TRAP assay. However, the ox-LDL lipid moiety could also participate to such an increased LDL-TRAP through their enriched-triglyceride content [50], and our \textit{in vitro} experiments confirmed that the addition of triglyceride-enriched LDL preparations significantly increased LDL-TRAP. The presence of elevated circulating levels of triglycerides is a common feature of HD patients [51], but here again, triglycerides by themselves have no antioxidant effect. It is therefore highly probable that when incorporated into LDL particles triglycerides become an additional target of the peroxyl radicals involved in linoleic acid oxidation and in this manner delay the CL reaction.

It therefore appears obvious that TRAP is not a reliable parameter for assessing the TRAP of plasma or LDL in HD patients. The evaluation of TRAP in this clinical setting, which combines major oxidative stress with profound uremia-related biochemical disturbances, has nevertheless allowed us to highlight the potential effects of uric acid, triglycerides, and AOPP on peroxyl radical-mediated reactions.

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\section*{Appendix}

Abbreviations used in this article are: ABAP, 2,2'-azobis[2-amidinopropane] hydrochloride; AGEs, advanced glycation end-products; AOPPs, advanced oxidation product proteins; CL, chemiluminescence; CRF, chronic renal failure; DNPPh, 2,4-dinitrophenylhydrazine; GSH-Px, glutathione peroxidase; HD, hemodialysis; HOCl, hypochlorous acid; HSA, human serum albumin; LDL, low-density lipoprotein; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate; ox-LDL, oxidized low-density lipoprotein; t lag, lag time; TPTZ, 2,4,6-tripyridyl-s-triazine; TRAP, total peroxyl radical-trapping antioxidant potential.

\begin{table}[h]
\centering
\caption{Effect of uric acid and HSA-AOPP on plasma CL reaction and TRAP}
\begin{tabular}{llllll}
\hline
 & Control plasma & Control plasma supplemented with uric acid or AOPP & HD plasma \\
\hline
Uric acid $\mu$mol/liter & 245 \pm 15 & +100 & +200 & No & No & 432 \pm 18 \\
AOPP $\mu$mol/liter & 31 \pm 10 & No & No & +100 & +150 & 174 \pm 14 \\
\hline
\end{tabular}
\end{table}

Results of 5 experiments are shown (mean \pm sd). \(P\) values were by the Mann-Whitney U-test versus control plasma before supplementation.

\section*{References}


