

Reduced hemodialysis-induced oxidative stress in end-stage renal disease patients by electrolyzed reduced water

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Background. Increased oxidative stress in end-stage renal disease (ESRD) patients may oxidize macromolecules and consequently lead to cardiovascular events during chronic hemodialysis. Electrolyzed reduced water (ERW) with reactive oxygen species (ROS) scavenging ability may have a potential effect on reduction of hemodialysis-induced oxidative stress in ESRD patients.

Methods. We developed a chemiluminescence emission spectrum and high-performance liquid chromatography analysis to assess the effect of ERW replacement on plasma ROS (H_2O_2 and HOCl) scavenging activity and oxidized lipid or protein production in ESRD patients undergoing hemodialysis. Oxidized markers, dityrosine, methylguanidine, and phosphatidylcholine hydroperoxide, and inflammatory markers, interleukin 6 (IL-6), and C-reactive protein (CRP) were determined.

Results. Although hemodialysis efficiently removes dityrosine and creatinine, hemodialysis increased oxidative stress, including phosphatidylcholine hydroperoxide, and methylguanidine. Hemodialysis reduced the plasma ROS scavenging activity, as shown by the augmented reference H_2O_2 and HOCl counts ($R_{H_2O_2}$ and R_{HOCl} , respectively) and decreased antioxidative activity (expressed as total antioxidant status in this study). ERW administration diminished hemodialysis-enhanced $R_{H_2O_2}$ and R_{HOCl} , minimized oxidized and inflammatory markers (CRP and IL-6), and partly restored total antioxidant status during 1-month treatment.

Conclusion. This study demonstrates that hemodialysis with ERW administration may efficiently increase the H_2O_2 - and HOCl-dependent antioxidant defense and reduce H_2O_2 - and HOCl-induced oxidative stress.

Hemodialysis is often used for removal of excessive toxins, metabolic products, and blood components from patients with end-stage renal diseases (ESRD). However,

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Key words: hemodialysis, electrolyzed reduced water, reactive oxygen species, end-stage renal diseases.

Received for publication September 11, 2002
and in revised form December 19, 2002, and March 12, 2003
Accepted for publication March 28 2003

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dialysis itself may further contribute to atherosclerosis by oxidative stress, cytokine stimulation, and other events inherent to hemodialysis [1–4]. The extracorporeal treatment per se is associated with imbalance between excess production of reactive oxygen species (ROS) reflected by increased serum indices of lipid peroxidation by monocytes and polymorphonuclear leukocytes [2, 3], protein products and a reduction in plasma antioxidants [5]. The increased oxidative stress may partially result from activation of leukocytes interacting with the dialyzer artificial membrane [3] and can cause oxidation of biologic macromolecules, including proteins and lipids [6, 7]. Among these molecules, oxidized low-density lipoprotein (LDL) can increase adhesion of monocytes to the endothelium and transformation of macrophages into foam cells and impair endothelium-dependent vasorelaxation [8–10]. These changes could lead to the development of atherosclerosis and coronary artery disease [9–11].

The two major ROS generated from activated neutrophils via the myeloperoxidase (MPO) system are hydrogen peroxide (H_2O_2) and hypochlorite (HOCl) [12]. The primarily sensitive and specific product of lipid peroxidation that can readily be measured in the laboratory is phosphatidylcholine hydroperoxide (PCOOH) [13]. Two other oxidized products, dityrosine and methylguanidine (MG), were also widely used as indirect indicators for ROS and/or free radical activities [14, 15].

Electrolyte reduced water (ERW) obtained by electrolysis scavenges ROS and protects DNA from oxidative damage [16]. The protective mechanism of ERW results from active atomic hydrogen with high reducing ability, which can contribute to ROS scavenging activity, and may participate in the redox regulation of cellular function [16]. Hemodialysis produces ROS in blood during dialysis and imposes heavy burden on patients; therefore, for decreases of hemodialysis-enhanced oxidative stress, we have considered administering ERW by a new setup of HD-24K (Nihom Trim Co., Osaka, Japan) to the patients during hemodialysis course. Three months

of hemodialysis session were used in this study as in vivo system to evaluate the effect of ERW on dialysis-induced oxidative stress. We clarified the response of $H_2O_2/HOCl$ system and to identify which ROS is scavenged by hemodialysis session with ERW administration. We used a characteristic emission spectrum analysis of the chemiluminescence (CL) spectrum for the first time to evaluate the specific ROS activity, including H_2O_2 and HOCl in the plasma and adapted a CL high-performance liquid chromatography (CL-HPLC) for measurement of PCOOH before and after the dialysis session, in the absence and presence of ERW. Our study showed that ERW administration partially restored ROS scavenging activity for H_2O_2 and HOCl. This treatment also resulted in lower plasma levels of oxidized lipids and proteins, and inflammatory markers in ESRD patients as compared to those before ERW treatment. Dialysate with simultaneous ERW replacement might be of clinical importance for preventing oxidative stress induced cardiovascular events in ESRD patients undergoing chronic hemodialysis.

METHODS

Patients

Ten healthy volunteers (three women and seven men; mean \pm SD; age 45 ± 12 years; range, 38 to 60 years) with cholesterol 189 ± 11 mg/dL and creatinine 1.0 ± 0.1 mg/dL were enrolled in this study. To assess the extent of oxidative stress in blood of ESRD patients, 37 patients (22 males and 15 females) with 10.3 ± 0.6 mg/dL of creatinine, subjected to maintenance hemodialysis at Wan-Hwa Hospital for a period of 12 ± 2 months, after informed consent was obtained, were enrolled in this study. Criteria for patient selection included the absence of habit of smoking, malignancy, inflammatory disorders, chronic or acute infections, supplementation of vitamin C or E, and treatment either oral or intravenous iron, or anti-inflammatory drugs 3 months before enrollment. The mean age was 63 ± 2 years old (mean \pm SEM, range 46 to 81 years). All hemodialysis patients received a standard bicarbonate dialysis schedule of 4 hours, three times a week, using a single use of Hemodialyzer (AF-150 and AF-180 membrane type; Althane, Althin Medical, Inc., Miami, FL, USA). The blood flow rate during the dialysis sessions was 250 to 300 mL/min and dialysate flow was 500 mL/min. The surface area of these two dialyzers was 1.5 m^2 and 1.8 m^2 respectively, and patient treatment with either membrane lasted for 6 months. The Kt/V of all these patients was maintained between 1.3 and 1.6. The cause of ESRD was diabetes in 18 patients and chronic glomerulonephritis in 19 patients. Hemodialysis patients were on a free diet with a normal and constant intake of essential fatty acids, and none of them took any drug with established or potential oxidizing effect or any antioxidants such as vitamin C or E

during the 3 months of the testing period. The permission for a clinical trial was approved by the Human Research Committee of the Wan-Hwa Regional Hospital.

Structure of HD-24K and electrolyzed reduced water

HD-24K (Nihom Trim Co.) was composed of an electric power supply unit for converting business purpose power supply to direct current power supply, an indication/operation unit that indicated and controlled the operation of the device, a control unit that input and output the data between the whole bodies of the device, and a personal computer with a remote control (Fig. 1). In addition, the unit adopts the original constant current system at the power supply unit to prevent the difference of electrolysis current occurred by the difference of electronic conductivity of the supplied water.

Raw water was supplied from "in" after being compressed by the compression pump and supplied into a compartment for electrolysis through a solenoid valve, which opened and closed synchronizing with the signal of water level sensor in electrolyzed water storage tank, flow sensor, and cross line "in" (watercourse exchanging unit). At the electric compartment, the supplied raw water was electrolyzed by direct current supplied to + and - electrode plate (Type TI-7000S and TI-7000SL, Nihon Trim Co.) across the diaphragm and then, at the side of - electrode plate, reduced water containing active hydrogen, and at the side of + electrode, plate acidic water, were collected from "out" and "drain" through cross-line "out" (watercourse exchanging unit), respectively. The series of operation were automatically done according to the level of reduced water tank.

The example of the system in which HD-24K is applied the hemodialysis system is indicated in Figure 1. HD-24K, compression pump, reduced water tank, acidic water tank, and personal computer for control were placed in front of the usual raw water processing device. Moreover, for the HD-24K system, an unused pipe arrangement and valve for bypass were installed. The reduced water produced by HD-24K was collected in the reduced water tank, and then was supplied to usual raw water processing system for hemodialysis by compression with the compressing pump.

The pH of electrolyzed-reduced water correspondence to the level for adjusting the electrolysis intensity, which showed in the example of a system in 5, is indicated as follows (the water right after collecting from "out" of HD-24K): level 1, pH = 9.5; level 2, pH = 9.8; level 3, pH = 10.0; and level 4, pH = 10.1. The intensity of electrolysis of HD-24K was adjusted to "level 2" and the ERW that indicated pH 9.8 right after taking from "out" of HD-24K was used in this study. The quality of ERW for hemodialysis was approved by Yen Tjing Ling Industrial Research Institute, National Taiwan University, with the No. E89A299D277, which is required by the

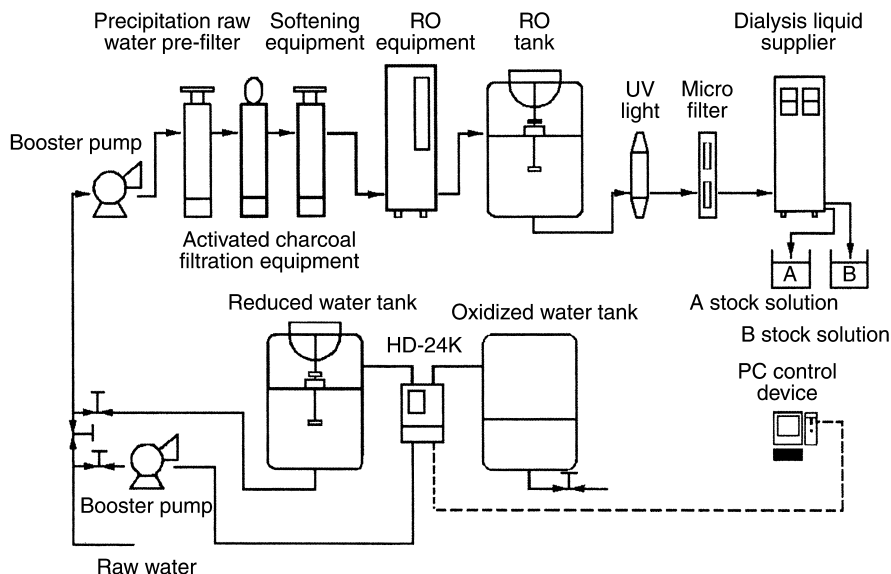


Fig. 1. The system in which HD-24K is applied to hemodialysis system is indicated. HD-24K, compression pump, reduced water tank, acidic water tank, and personal computer for control are placed in front of usual raw water processing device. The intensity of electrolysis of HD-24K is adjusted to "level 2" and the electrolyzed reduced water (ERW) that indicates pH 9.8 right after taking from "out" of HD-24K is used in this study.

American Association of Medical Instruments (AAMI). The endotoxin level in the water used for hemodialysis is 0.069 ± 0.004 EU/mL measured by an endotoxin enzyme-linked immunosorbent assay (ELISA) kit (HBT-HIT301, Biocompare Inc., South San Francisco, CA, USA) and an automated ELISA analyzer (CODA, SN10373, Hercules, CA, USA).

Blood samples and biochemical analysis

Ten milliliters of blood were drawn from the arterial side of the arteriovenous fistula before and after dialysis in hemodialysis patients. Then blood was collected into heparinized sterile test tubes and processed within 2 hours. Plasma was separated from blood cells by centrifugation at $1500 \times g$ for 5 minutes at 4°C . The plasma was immediately stored at -70°C and analyzed within 2 weeks.

Plasma total cholesterol, triglycerides, and high-density lipoprotein (HDL) levels were assessed enzymatically by automated procedures. LDL, very low-density lipoprotein (VLDL), and cholesterol values were measured as previously described [17]. The measurement of lipoprotein(a) [Lp(a)] was performed with a commercial LPA kit (Kit Recorder #465360, Beckman Coulter Array System, Denmark). Quantitative determination of fibrinogen levels in plasma was made by FIBRI-PREST[®] AUTOMATE (Diagnostica Stago, Asnieres-Sur-Seine, France).

Red and white blood cells, hemoglobin level, hematocrit level, and platelets were determined by an autoanalyzer (Coulter STKS, Hialeah, Finland). Plasma total protein, albumin, urea nitrogen, creatinine, and alkaline phosphatase levels were determined by an autoanalyzer (Hitachi 736-15, Ibaraki, Japan).

Total antioxidant status (TAS) assay

The total antioxidant status (TAS) in $20 \mu\text{L}$ of plasma, ERW, or distilled water (as a blank) was measured with a TAS kit (Cat. # NX2332, Randox, San Francisco, CA, USA) according to the manufacturer's instructions. Briefly, $20 \mu\text{L}$ of sample was added to 1 mL of $6.1 \mu\text{mol/L}$ metmyoglobin and $610 \mu\text{mol/L}$ ABTS[®] (2,2'-Azino-di-3-ethylbenzthiazoline sulphonate). After thorough vortexing, the absorbance of the mixture was measured at 600 nm by an autoanalyzer (Tectron U-240 Plus, Japan). 6-Hydroxy-2,5,7,8-tetramethylchromon-2-carboxylic acid was used a standard. The assay principle was that the ABTS[®] was incubated with a peroxidase and H_2O_2 to produce the radical cation ABTS[®]. It has a relatively stable blue-green color, which was measured at 600 nm . Antioxidants in the added sample caused suppression of this color production to a degree, which was proportional to their concentration.

Measurement of specific plasma antioxidant activity

CL signals emitted from the "test mixture" of plasma [or phosphate-buffered saline (PBS) (50 mmol/L , pH 7.4) as a background control], H_2O_2 (or HOCl), and CL-emitting substance [i.e., luminol (5-amino-2,3-dihydro-1,4-phthalazinedione); Sigma, Chemical Co., St. Louis, MO, USA] was measured with a multiwavelength CL spectrum analyzer (CLA-SP2, Tohoku Electronic Ind., Co., Sendai, Japan).

We noted that the CL signals emitted from the H_2O_2 -luminol-plasma mixture (or HOCl-luminol-plasma mixture) decreased as the volume of added plasma in the test mixture rose (Fig. 2). In this study, we used $25 \mu\text{L}$ of plasma sample or $25 \mu\text{L}$ of PBS throughout. We first mixed $25 \mu\text{L}$ of plasma and 1.0 mL of $25 \mu\text{mol/L}$ luminol

in a 4.0 mL quartz cell ($1 \times 1 \times 4$ cm) for 100 seconds. Next, 1.0 mL of 0.03% H_2O_2 or 0.012% NaOCl was immediately added into the quartz cells. Luminol stock solution (250 $\mu\text{mol/L}$) was prepared as 1 mg of luminol dissolved in 22.7 mL of PBS.

The CL emitted from the above reaction mixture was recorded and measured as “reference H_2O_2 counts” (RH_2O_2) or “reference HOCl counts” (RHocl). Plasma from healthy donors was used for measurement of normal RH_2O_2 and RHocl . PBS was added to the test system, and the RH_2O_2 and RHocl yielded were recorded as the background counts (i.e., total RH_2O_2 and RHocl without inhibition by plasma). A higher RH_2O_2 or RHocl indicated lower antioxidative activity and/or lower ROS scavenging ability.

Using the above system, we also tested antioxidant activity of vitamin C (1 mg/mL), a scavenger for H_2O_2 [18], and (–) epigallocatechin-3-gallate (EGCG) (1 mg/mL), a scavenger for HOCl [19], ERW collected from extracorporeal system, PBS, and normal plasma on the response of RH_2O_2 or RHocl .

Measurement of oxidized protein/amino acid products

In the presence of H_2O_2 , the enzyme MPO can generate tyrosyl radicals by abstraction of H^\bullet from the –OH group on tyrosine, and the tyrosyl radicals may participate in the oxidation of LDL [20]. When a tyrosyl radical is generated in biologic systems, it often cross links to give a fluorescent adduct, dityrosine, which can be determined with a fluorometer (Hitachi F-2500, Tokyo, Japan), as described previously [15]. The sample (5 μL of serum in 1 mL of distilled water) was measured at an excitation wavelength of 315 nm and an emission wavelength of 410 nm.

The hydroxyl radical, an important product of ROS, is known to play a role in the biosynthesis of MG, which contributes to toxicity in uremic patients. The MG with fluorescent activity, as an indirect measure of hydroxyl radical activity, was determined as described previously [16]. The sample was assayed at an excitation wavelength of 395 nm and an emission wavelength of 500 nm. The concentrations of dityrosine or MG were displayed as fluorescence intensity/mL (FI/mL).

Measurement of lipid peroxidation products

For measurement of oxidation of LDL, the competition ELISA method is developed to detect malondialdehyde (MDA)-LDL and oxidized LDL in circulating plasma using monoclonal antibodies 4E6 and 1H11 [21]. Both antibodies 4E6 and 1H11 react not only to oxidized LDL but also to MDA-LDL. Because MDA is one of those lipid peroxidation products that is highly sensitive reactive to lysine residues, MDA-LDL has been widely used as a way to detect and quantify oxidized LDL. However, despite the fact that oxidized LDL contains

MDA-induced modifications of the apolipoprotein B (apoB) protein, MDA-LDL cannot be considered identical to oxidized LDL [22]. Watson et al [23] demonstrated that oxidized phosphatidylcholine (PC) separated from minimally modified LDL, prepared by mild oxidation of LDL, was capable of inducing monocyte adhesion to endothelial cells and neutrophil migration. They suggest that oxidized PC is one of the key molecules in oxidized LDL and is directly involved in the early development of atherosclerosis. Therefore, in this experiment, measuring oxidized LDL in circulating plasma using PCOOH, a primary lipid peroxidation products, could provide a means to monitor the behavior of oxidized PC particles as part of oxidized LDL in plasma LDL fractions.

The amounts of PCOOH were determined in duplicate by CL-HPLC (Tohoku Electronic Ind., Co.) [13]. Briefly, the total lipids were extracted from 0.5 mL of plasma, with 2 mL of chloroform and methanol mixture (2:1, vol/vol, containing 0.002% butylated hydroxytoluene as an antioxidant) added, followed by vigorous mixing. The extraction was repeated and the chloroform layer collected. After dehydration and evaporation of the combined chloroform layer, the dried total lipid residue was diluted with 40 μL of chloroform-methanol (2:1, vol/vol) and a 20 μL portion was used for CL-HPLC. CL was produced through luminol oxidation during the reaction between PCOOH and cytochrome c.

Interleukin-6 (IL-6) and C-reactive protein (CRP) assay

In the present study, cytokine release [interleukin-6 (IL-6)] and subsequent production of acute phase proteins [C-reactive protein (CRP)] were assessed to investigate whether the ERW dialysate can ameliorate hemodialysis-enhanced CRP and IL-6 levels. For in vitro use with the IMMULITE Analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA), the quantitative measurement of IL-6 (LK6PZ) by sequential immunometric assay in heparinized plasma was performed as an aid in the study of inflammatory diseases. Samples were analyzed in duplicate, according to the manufacturer's instructions. The lower limit of detection was 5 pg/mL. Serum CRP concentrations measured by an immunometric kit (717-80A3, Iatron Laboratories, Inc., Tokyo, Japan) were determined by an autoanalyzer (Tectron U-240 Plus). The lower limit of detection of CRP was 0.3 mg/L.

Statistical analysis

All values are expressed as mean \pm SEM, and $P < 0.05$ was considered to indicate statistical significance. The values were analyzed by *t* test and analysis of variance (ANOVA) for repeated measures where appropriate.

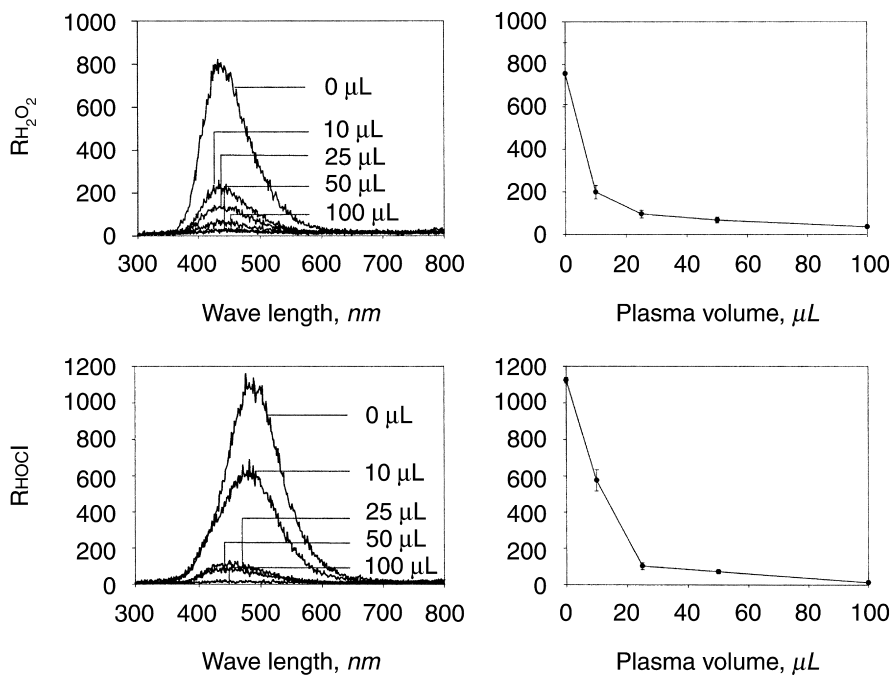


Fig. 2. Typical emission spectra of the chemiluminescence (CL) of a test mixture containing H_2O_2 and luminol for reference H_2O_2 (RH_2O_2), HOCl, and luminol for reference HOCl (RHocl) and different volumes of plasma or [phosphate-buffered saline (PBS)] obtained from a healthy control. Increased plasma volume (from 0 to 100 μL) added to the test mixture could lower both RH_2O_2 and RHocl counts. Mean change of RH_2O_2 and RHocl counts with different plasma volume added to the test mixture was displayed in the right panels.

RESULTS

ERW exerted potent antioxidant activity for RH_2O_2 and RHocl

Figure 2 shows a typical CL emission from H_2O_2 (counts/300 seconds) in the presence of a plasma sample (or PBS), luminol, and 0.03% H_2O_2 . The H_2O_2 CL has a wide emission wavelength region of 370 to 650 nm with an emission maximum of 460 nm. The CL count read at emission maximum was expressed as RH_2O_2 . The background RH_2O_2 in the PBS-luminol-0.03% H_2O_2 mixture was 756 ± 145 counts. When increased volume of plasma from healthy individuals was added, the RH_2O_2 decreased to 198 ± 32 counts, 95 ± 18 counts, 64 ± 14 counts, and 35 ± 3 counts (100 μL , 50 μL , 25 μL , and 10 μL , respectively) indicating the presence of H_2O_2 scavenger activity in plasma (Fig. 2).

The HOCl CL has a wide emission wavelength region of 350 to 670 nm with an emission maximum of 520 nm (Fig. 2). The RHocl in the PBS-luminol-0.012% HOCl mixture was 1124 ± 16 counts. When different volume of control plasma was added, the RHocl was reduced to 576 ± 59 counts, 101 ± 19 counts, 71 ± 10 counts, and 13 ± 1 counts (100 μL , 50 μL , 25 μL , and 10 μL , respectively) also indicating the presence of HOCl scavenger activity in plasma (Fig. 2).

Ascorbate (1 mg/mL in 25 μL), EGCG (1 mg/mL in 25 μL), ERW (25 μL), and normal plasma (25 μL) added to the test mixture revealed strong H_2O_2 scavenger activity in an order EGCG = ascorbate > normal plasma > ERW \gg PBS, as shown by a reduction in RH_2O_2 (Fig. 3A). Ascorbate, EGCG, normal plasma, and ERW

seem to be effective scavengers for RH_2O_2 . However, ascorbate (1 mg/mL in 25 μL), EGCG (1 mg/mL in 25 μL), ERW (25 μL), and normal plasma (25 μL) added to the test mixture revealed HOCl scavenger activity in an order EGCG > normal plasma > ERW \gg PBS = ascorbate, as shown by a reduction in RHocl (Fig. 3B). EGCG, normal plasma, and ERW seem to be effective scavengers for RHocl , but not ascorbate. ERW displayed antioxidant activities for both H_2O_2 and HOCl.

ERW reduced hemodialysis-enhanced RH_2O_2 and RHocl counts

In Figure 4, typical emission of RH_2O_2 and RHocl from one ESRD patient was displayed before ERW treatment, and during initial and 1-month ERW treatment. We noted that prehemodialysis value of RH_2O_2 and RHocl was similar in the group without ERW treatment and ERW-treated group. Response of RH_2O_2 and RHocl in post-hemodialysis plasma was significantly increased when compared to respective prehemodialysis value of RH_2O_2 and RHocl . However, the enhanced response of RH_2O_2 and RHocl was partly decreased by ERW treatment.

As shown in Figure 5, the mean value of RH_2O_2 and RHocl was 87 ± 14 and 65 ± 10 counts in nonhemodialysis plasma from 10 healthy individuals. The prehemodialysis value of RH_2O_2 and RHocl was 154 ± 18 and 97 ± 16 counts, respectively, in 37 ESRD patients' plasma without ERW treatment. This data directly indicate that a significantly increased oxidative stress occurred in ESRD patients. After single hemodialysis course without ERW treatment, both RH_2O_2 and RHocl counts were significantly increased to 416 ± 46 and 210 ± 17 counts

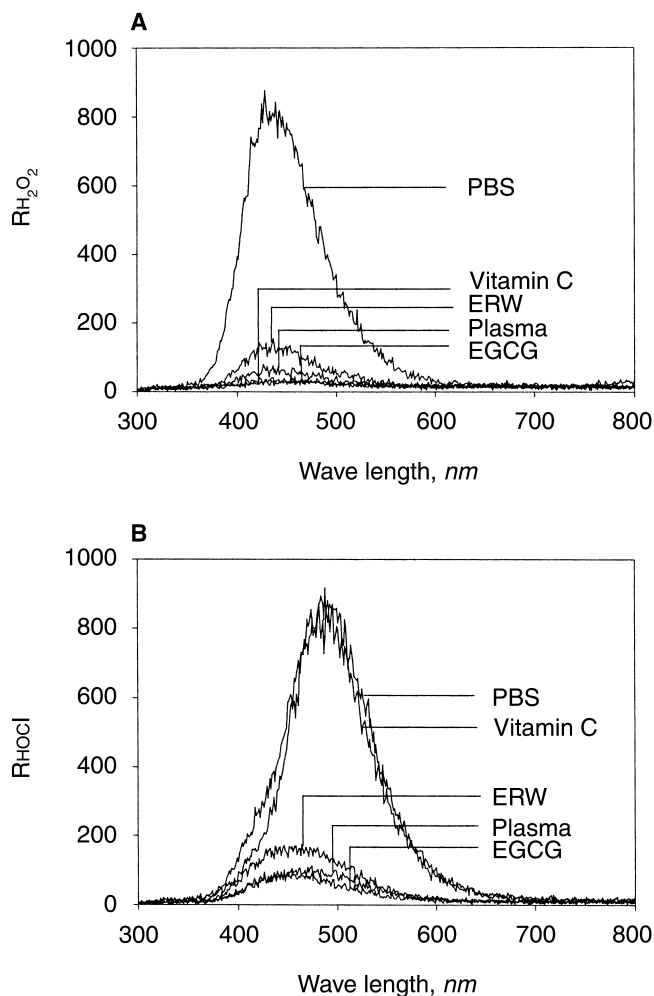


Fig. 3. Typical emission spectra of the reference H_2O_2 (RH_2O_2) (A) and reference HOCl (RHocl) (B) counts with various kinds of antioxidants [vitamin C, epigallocatechin-3-gallate (EGCG), and electrolyzed reduced water (ERW)], plasma from healthy subjects and phosphate-buffered saline (PBS) are displayed. Vitamin C, ERW, plasma, and EGCG significantly reduced RH_2O_2 counts, whereas ERW, plasma, and EGCG, but not vitamin C, decreased RHocl counts.

($P < 0.05$), respectively, when compared to their pre-hemodialysis values (154 ± 18 and 97 ± 16 counts).

After initial and 1-month ERW treatment, the pre-hemodialysis values of RH_2O_2 and RHocl in the ESRD plasma were not significantly affected when compared to those in without ERW treatment (RH_2O_2 , 154 ± 18 counts in the group without ERW treatment vs. 158 ± 23 counts in initial ERW treatment and 174 ± 9 counts in 1-month ERW treatment, respectively; RHocl , 97 ± 16 counts vs. 92 ± 23 counts in initial ERW treatment and 87 ± 21 counts in 1-month ERW treatment, respectively). However, the posthemodialysis values in RH_2O_2 and RHocl were significantly reduced by ERW treatment after initial (304 ± 16 counts in RH_2O_2 and 154 ± 11 counts in RHocl) and 1-month (262 ± 31 counts in RH_2O_2 and 134 ± 7 counts in RHocl) of ERW treatment ($P < 0.05$).

ERW partly restored TAS

The effect of reducing oxidative stress by ERW was further confirmed by the mildly increased TAS in pre-hemodialysis plasma and partly restored in posthemodialysis plasma after 1-month ERW treatment (Fig. 6B). The plasma TAS was significantly higher in healthy controls than in ESRD patients. Hemodialysis reduced the plasma TAS, and the reduction could be partially restored by 1-month ERW administration. More significantly, ERW appeared markedly to enhance the scavenging activity for H_2O_2 and HOCl (Fig. 5).

Effects of ERW on biochemical parameters

A single session of hemodialysis with or without ERW treatment similarly decreased body weight (from 63.1 ± 1.91 kg to 59.9 ± 1.83 kg), blood urea nitrogen (from 70.8 ± 5.4 mg/dL to 21 ± 2.1 mg/dL), and creatinine (from 12.3 ± 0.6 mg/dL to 5.1 ± 0.4 mg/dL), increased white blood cell (from 5842 ± 287 μL to 6108 ± 332 μL), increased hemoglobin (from 10.9 ± 0.40 g/dL to 12.6 ± 0.50 g/dL), increased hematocrit (from $32.2 \pm 1.2\%$ to $37.0 \pm 1.5\%$), and increased platelets (from $171 \pm 8 \times 10^3/\mu\text{L}$ to $201 \pm 8 \times 10^3/\mu\text{L}$) in 37 ESRD patients. The prehemodialysis and posthemodialysis levels of LDL, VLDL, and Lp(a) with or without ERW treatment are displayed in Table 1.

The levels of lipid-peroxidation primary products (PCOOH), and protein/amino acid oxidation products (MG and dityrosine) in ESRD patients before and after hemodialysis with and without ERW treatment are displayed in Figure 6.

In ESRD patients, the levels of PCOOH (192 ± 18 ng/mL), MG (529 ± 48 FI/mL), and dityrosine (7404 ± 359 FI/mL) were significantly higher than those in healthy controls (PCOOH, 44 ± 10 ng/mL; MG, 237 ± 41 FI/mL; and dityrosine, 1658 ± 232 FI/mL) ($P < 0.05$), also indicating an increased oxidative stress status in ESRD condition. After hemodialysis without ERW treatment, the levels of PCOOH were increased significantly ($P < 0.05$) (from 192 ± 18 ng/mL to 652 ± 76 ng/mL), but dityrosine decreased after hemodialysis session (from 7404 ± 359 FI/mL to 5118 ± 313 FI/mL) ($P < 0.05$). MG was only marginally increased after hemodialysis without ERW (from 529 ± 48 FI/mL to 548 ± 53 FI/mL). With initial and 1-month ERW administration, the posthemodialysis PCOOH levels were significantly reduced (190 ± 18 ng/mL and 335 ± 47 ng/mL), whereas the posthemodialysis levels of MG and dityrosine were affected marginally. The mild reduction in MG or dityrosine in posthemodialysis plasma was found after 1-month ERW treatment. PCOOH levels after 1-month ERW treatment are higher than single treatment with ERW. This discrepancy may be due to a dietary factor that increased the levels in triglyceride

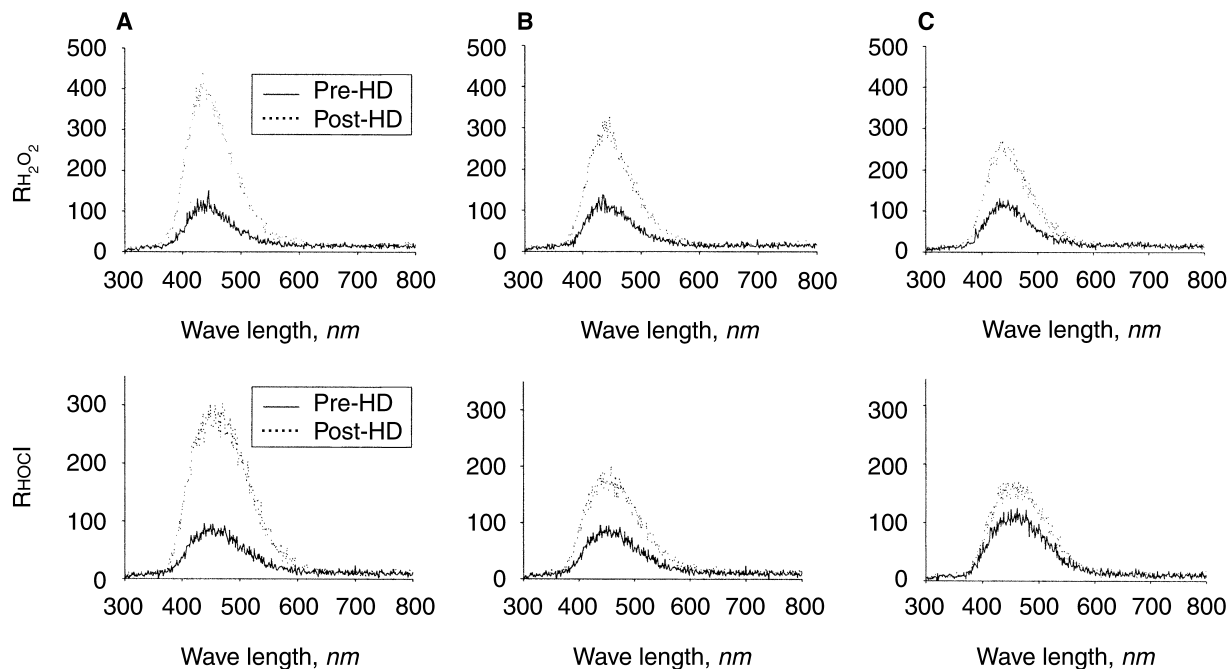


Fig. 4. Hemodialysis (HD) effect on emission spectra of the reference H_2O_2 (RH_2O_2) and reference HOCl (RHocl) counts of prehemodialysis and posthemodialysis plasma from one end-stage renal disease (ESRD) patient before electrolyzed reduced water (ERW) treatment (A), after initial ERW (B), and 1-month ERW (C) treatment are displayed. Before ERW treatment, the postdialysis plasma had significantly elevated RH_2O_2 and RHocl counts when compared to each prehemodialysis value. Initial and 1-month ERW supplement resulted in augmented reactive oxygen species (ROS) scavenging activity for both H_2O_2 and HOCl, as shown by the marked reduction of RH_2O_2 and RHocl counts in posthemodialysis plasma.

and VLDL in the ESRD patients with 1-month ERW treatment (Table 1).

As shown in Table 1, the prehemodialysis level of CRP and IL-6 was efficiently ($P < 0.05$) reduced with ERW treatment in 10 ESRD patients with detectable CRP and IL-6 levels.

DISCUSSION

Hemodialysis efficiently removed blood urea nitrogen and creatinine, but had no effects on atherogenic substances, such as total LDL, cholesterol, plasma Lp(a), fibrinogen, and oxidized products from hemodialysis patients. Hemodialysis appears to cause an acute and transient reduction in endogenous antioxidant ability, as shown by the decrease in TAS (our data) and increased production of ROS by leukocytes after hemodialysis. As a result, there was an increase in patients' plasma RH_2O_2 and RHocl after hemodialysis. ERW administration during hemodialysis session partly restored TAS and efficiently diminished RH_2O_2 and RHocl . More significantly, the level of oxidative products of lipid and protein can be partly reduced by ERW supplement, despite the oxidative stress accompanying hemodialysis.

The increased ROS found in patients with ESRD undergoing chronic hemodialysis could originate from complement-, platelet-, and even dialysis membrane-activated leukocytes [24, 25]. The self-perpetuating formation of H_2O_2

and HOCl from leukocytes may potentially oxidize protein and LDL, change the lipid composition of cell membranes and the extracellular matrix [26, 27], and consequently lead to atherogenic injury. Cells (such as endothelial cells) are more prone to oxidative damage when exposed to H_2O_2 as compared to HOCl [28]. However, when combined with H_2O_2 , HOCl increased H_2O_2 -mediated oxidative damage and compromised the repair process. Increased plasma H_2O_2 by reducing the bioavailability of nitric oxide (NO) and impairing normal platelet inhibitory mechanisms may cause thrombotic disorder in humans [29]. Other data also implicate that cardiopulmonary bypass activates complement via the alternate (C3a) pathway and increases plasma H_2O_2 by pulmonary sequestration of polymorphonuclear leukocytes [30]. Activated phagocyte cells generate hypochlorite (HOCl) via the release of H_2O_2 and the enzyme MPO. Plasma proteins are major targets for HOCl. The reaction of fresh diluted plasma with HOCl gives rise to protein-derived nitrogen-centered radicals in a time- and HOCl concentration-dependent manner; these have been detected by electron paramagnetic resonance (EPR) spin trapping. Identical radicals have been detected with isolated HOCl-treated plasma proteins [31]. Thus, antioxidant treatment that can reduce HD-induced H_2O_2 and HOCl should be beneficial for minimizing oxidative damage to leukocytes and endothelial cells.

Based upon the interesting clinical improvement of a

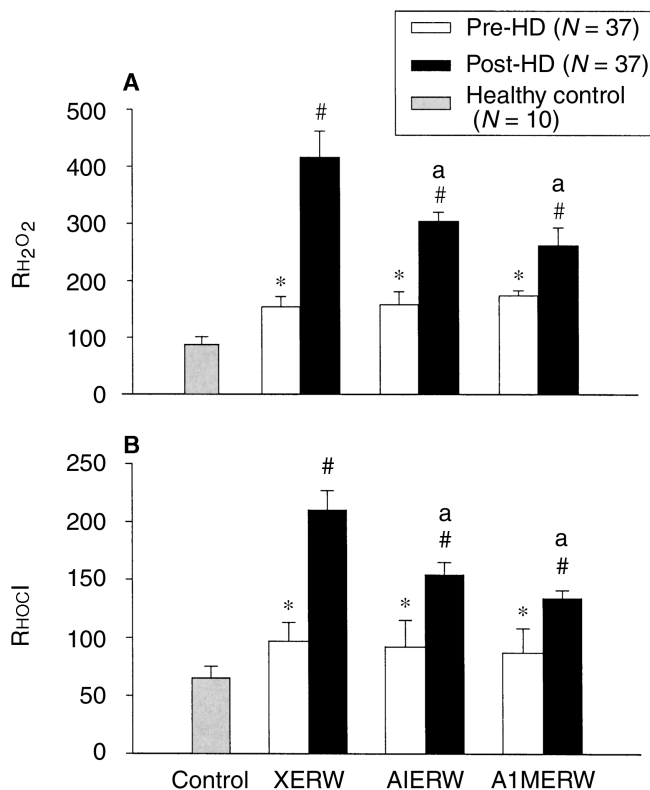


Fig. 5. Effects of hemodialysis (HD) and electrolyzed reduced water (ERW) supplement on reference H₂O₂ (RH₂O₂) (A), and reference HOCl (RHocl) (B) counts in plasma obtained from 37 end-stage renal disease (ESRD) patients. The levels of RH₂O₂ and RHocl of prehemodialysis plasma in 37 ESRD patients were higher than those in 10 healthy control subjects. The posthemodialysis plasma had elevated RH₂O₂ and RHocl. However, after initial (A1ERW) and 1-month ERW (A1MERW) supplementation resulted in augmented reactive oxygen species (ROS) scavenging activity for both H₂O₂ and HOCl, as shown by the marked reduction of RH₂O₂ and RHocl. **P* < 0.05 the prehemodialysis values of ESRD plasma vs. the values of healthy control plasma; #*P* < 0.05 the posthemodialysis values vs. the prehemodialysis value of the respective group. ^a*P* < 0.05 when compared to the value of ESRD plasma without ERW (XERW) administration.

variety of diseases by intake of reduced water since 1985, Hayashi [32] proposed the hypothesis “water regulating theory.” The ideal scavenger for ROS should be “active atomic hydrogen.” “Active atomic hydrogen” can be produced in reduced water near the cathode during electrolysis of water. Reduced water exhibits high pH, low dissolved oxygen, extremely high dissolved molecular hydrogen, and extremely negative redox potential values [16]. Shirahata et al [16] suggest that the superoxide dismutase (SOD)- and catalase-like activity of ERW is not due to the dissolved molecular hydrogen but due to the active atomic hydrogen with a higher reducing ability that may participate in ROS scavenging activity. Happe et al [33] indicate that hydrogenases, which are among in the oldest enzymes (3.8 billion years old), can reversibly split molecular hydrogen to produce active atomic hydrogen, which participates in the redox regulation of cellular

Table 1. Effect of electrolyzed reduced water (ERW) on the levels of low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), lipoprotein (a) [Lp(a)], interleukin-6 (IL-6), and C-reactive protein (CRP) in end-stage renal disease (ESRD) patients before and after hemodialysis treatment

Number	Triglyceride mg/dL		LDL mg/dL		VLDL mg/dL		Lp(a) mg/dL		IL-6 mg/dL		CRP mg/dL	
	Pre-HD	Post-HD	Pre-HD	Post-HD	Pre-HD	Post-HD	Pre-HD	Post-HD	Pre-HD	Post-HD	Pre-HD	Post-HD
XERW	136 ± 57	114 ± 50	73 ± 5	98 ± 6 ^a	11.4 ± 2.2	10.9 ± 2.1	7.9 ± 1.9 (10)	6.5 ± 1.0 (10)	0.72 ± 0.28 (10)	0.69 ± 0.16 (10)	0.72 ± 0.28 (10)	0.69 ± 0.16 (10)
A1ERW	141 ± 30	142 ± 40	83 ± 8	106 ± 10 ^a	12.6 ± 2.9	11.9 ± 2.9	7.8 ± 0.8 (10)	5.7 ± 0.7 ^a (10)	0.45 ± 0.20 ^b (10)	0.47 ± 0.21 (10)	0.45 ± 0.20 ^b (10)	0.47 ± 0.21 (10)
A1MERW	170 ± 35	209 ± 49	79 ± 6	104 ± 9 ^a	11.6 ± 2.8	10.3 ± 3.1	5.7 ± 0.7 ^b (10)	5.4 ± 0.4 (10)	0.39 ± 0.16 ^b (10)	0.36 ± 0.16 (10)	0.39 ± 0.16 ^b (10)	0.36 ± 0.16 (10)

Data is expressed as mean ± SE. Abbreviations are: pre-HD, before hemodialysis; post-HD, after hemodialysis; XERW, without ERW treatment; A1ERW, after initial ERW treatment; A1MERW, after 1-month ERW treatment; (10), 10 ESRD patients with detectable CRP levels.
^a*P* < 0.05 vs. pre-HD value of respective group
^b*P* < 0.05 vs. pre-HD value of XERW group

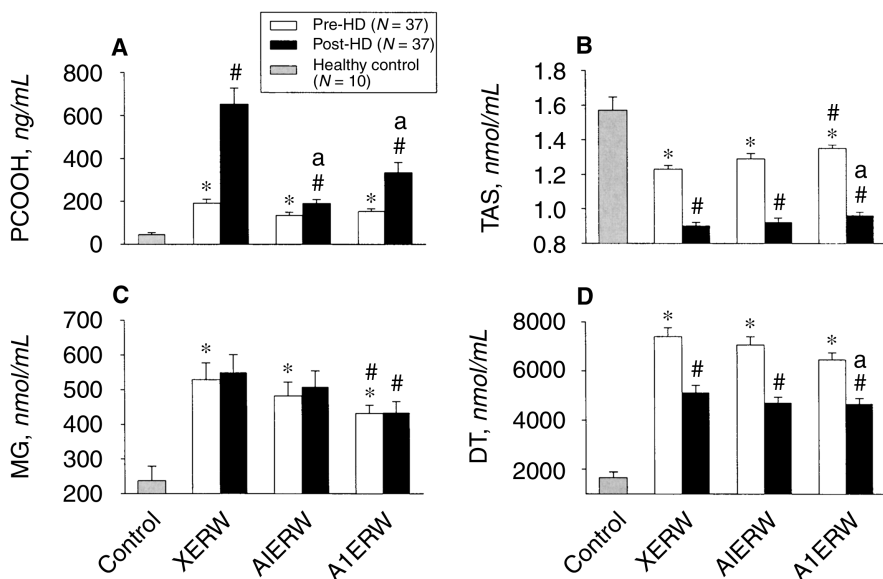


Fig. 6. Effects of hemodialysis (HD) and electrolyzed reduced water (ERW) supplement on PCOOH (A), total antioxidant status (TAS) (B), methylguanidine (MG) (C), and dityrosine (DT) (D) in plasma obtained from 37 end-stage renal disease (ESRD) patients. The levels in PCOOH, MG, and DT of prehemodialysis plasma in 37 ESRD patients without ERW treatment (XERW) were higher than those in 10 healthy control subjects. A significant decrease in TAS of prehemodialysis plasma was found in ESRD patients. When compared to prehemodialysis value, the posthemodialysis plasma had significantly elevated PCOOH levels and marginally increased MG and decreased TAS and DT values. Initial ERW (A1ERW) supplementation significantly reduced PCOOH levels, but did not significantly affect TAS, MG, and DT values. However, 1-month ERW (A1MERW) supplementation mildly restored TAS ability in posthemodialysis plasma and consequently mildly reduced PCOOH, MG, and DT levels in posthemodialysis plasma. The level of PCOOH in posthemodialysis plasma after 1-month ERW treatment was higher than that after initial ERW treatment possibly by a dietary factor. Note that a marginally increased TAS, and a decreased MG was found in prehemodialysis plasma after 1-month ERW treatment. * $P < 0.05$ the prehemodialysis values of ESRD plasma vs. the values of healthy control plasma. # $P < 0.05$ the posthemodialysis values vs. the prehemodialysis value of the respective group. ^a $P < 0.05$ when compared to the value of ESRD plasma without ERW (XERW) administration.

function. The ROS scavenging activity of ERW is stable at 4°C for over a month and was not lost even after neutralization, repeated freezing and melting, deflation with sonication, vigorous mixing, boiling, repeated filtration, or closed autoclaving, but was lost by opened autoclaving or by closed autoclaving in the presence of tungsten trioxide, which efficiently adsorbs active atomic hydrogen [16]. Strong ERW, as well as ascorbic acid and catechin, completely scavenged superoxide anion produced by the hypoxanthine-xanthine oxidase system in sodium phosphate buffer [16]. Furthermore, ERW, as well as catalase and ascorbic acid, could directly scavenge H_2O_2 and possible 1O_2 and $^{\bullet}OH$ [16]. In our experiment with CL spectrum, we found that ERW and EGCG can scavenge both H_2O_2 and HOCl, but ascorbic acid only available removes H_2O_2 . The scavenging ROS mechanism by ERW may be due to the direct adsorption of ROS by active atomic hydrogen, because no antioxidant production by TAS assay was detected in our experiment (data unrepresented). This result indicates that ERW with a higher reducing ability and/or direct ROS scavenging activity could be used in hemodialysis patients by its stable characteristics, cheaper expense, and strong antioxidant activity.

Hemodialysis does not efficiently and mechanically

remove atherogenic substances, such as total LDL, plasma Lp(a), cholesterol, fibrinogen, and oxidized products (PCOOH and MG) from ESRD patients. Furthermore, hemodialysis-induced oxidative stress may, at the same time, cause the production of oxidized lipid and protein products. Among these products, PCOOH, accumulated as a primary peroxidation product from membrane phospholipids, was found to be a more sensitive marker than MDA in reflecting lipid peroxidation [34]. Despite the fact that oxidized LDL contains MDA-induced modifications of the apoB protein, MDA-LDL cannot be considered identical to oxidized LDL [22]. Watson et al [23] also demonstrated that oxidized PC was capable of inducing monocyte adhesion to endothelial cells and neutrophil migration. They suggest that oxidized PC is one of the key molecules in oxidized LDL and is directly involved in the early development of atherosclerosis. PCOOH is mostly distributed in the VLDL and LDL fractions [9]. There was an increased fraction of VLDL and LDL and a significant enhancement in the PCOOH fraction after hemodialysis in ESRD patients without ERW treatment. ERW with antioxidant activity may reduce hemodialysis-induced oxidative stress and consequently decrease the PCOOH level in ESRD patients. The dialysis clearance of MG was found to be lower

during a hemodialysis course. Its protein binding, which rises as the plasma pH rises during hemodialysis, accounts for its dialysis behavior. The low dialysis clearance of MG explains why the postdialysis percentage decrease in its plasma levels is lower than that of urea and of creatinine [35]. The dialytic behavior of MG is different from that of urea and of creatinine, which have similar molecular weights and the conclusions drawn from the behavior of the latter two metabolites cannot be applied to MG. The slow transfer of MG from tissue during dialysis, shown by direct measurements on plasma of ESRD patients, accounts for its high plasma rebound level after hemodialysis. The increased PCOOH and MG level might reflect increased ROS production under pathologic conditions and even during hemodialysis courses. Simultaneous treatment with ERW effectively lowered the levels of PCOOH as well as those of other oxidized products.

In chronic hemodialysis, morbidity may result from repetitive induction of the acute phase response and chronic inflammation and cause by a bioincompatible dialysis membrane [36] and/or contaminated dialysate [37]. Cytokines released from jeopardized tissues stimulate the liver to synthesize acute phase proteins, including CRP. It has been suggested that CRP is useful not only as a marker of the acute phase response, but is also involved in the pathogenesis of the disease. CRP may directly interact with the atherosclerotic vessels or ischemic myocardium by activation of the complement system, thereby promoting inflammation and thrombosis. Several studies in uremic patients have implicated CRP as a marker of malnutrition, resistance to erythropoietin, and chronic stimulation in hemodialysis. An increased cytokine production secondary to blood interaction with bioincompatible dialysis components has been reported by several studies; interleukin-1, tumor necrosis factor- α , and mainly IL-6 are the three proinflammatory cytokines involved in the pathogenesis of hemodialysis-related disease. Panichi et al [37] have provided evidence for the occurrence of high CRP and IL-6 levels in chronic hemodialysis patients. Therefore, CRP is implicated as a marker linking bioincompatibility and increased cytokine IL-6 production with a clinical state of chronic inflammation [37]. The effects of initial ERW on most oxidative parameters (H_2O_2 , HOCl, and PCOOH) at posthemodialysis plasma are significant, but these effects cannot be seen at prehemodialysis plasma. The decrease in several oxidative parameters in ESRD patients by ERW replacement may functionally restore TAS, which can reflect the total effects on all parameters. Therefore, 1-month ERW treatment mildly increased TAS and efficiently reduced PCOOH, MG, and chronic inflammatory markers (CRP and IL-6) in the prehemodialysis plasma of ESRD patients, expecting that a long-term outcome of therapeutic potential by ERW treatment may gradually improve constitutionally ESRD patients.

CONCLUSION

We have shown that hemodialysis with ERW supplement might be of clinical importance for reduction in oxidized macromolecules in ESRD patients with chronic hemodialysis. ERW treatment and other measures that can reduce ROS, particularly H_2O_2 and HOCl, should be beneficial for minimizing oxidative damage to leukocytes and endothelial cells. The application of ERW in dialysate may efficiently ameliorate hemodialysis-enhanced oxidative stress.

ACKNOWLEDGMENTS

We thank Dr. Liou, Shaw-Yih for providing CLA-SP2 to measure oxidative stress in this work. This work was supported partly by NSC (91-2320-B002-088), the Formosan Blood Purification Foundation, and Hopping Hospital Blood Dialysis Center.

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