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Electrolyzed-reduced water reduced hemodialysis-induced erythrocyte impairment in end-stage renal disease patients

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Chronic hemodialysis (HD) patients increase erythrocyte susceptibility to hemolysis and impair cell survival. We explored whether electrolyte-reduced water (ERW) could palliate HD-evoked erythrocyte impairment and anemia. Forty-three patients undergoing chronic HD were enrolled and received ERW administration for 6 month. We evaluated oxidative stress in blood and plasma, erythrocyte methemoglobin (metHb)/ferricyanide reductase activity, plasma metHb, and proinflammatory cytokines in the chronic HD patients without treatment ($n = 15$) or with vitamin C (VC)- ($n = 15$), vitamin E (VE)-coated dialyzer ($n = 15$), or ERW treatment ($n = 15$) during an HD course. The patients showed marked increases (15-fold) in blood reactive oxygen species, mostly H_2O_2, after HD without any treatment. HD resulted in decreased plasma VC, total antioxidant status, and erythrocyte metHb/ferricyanide reductase activity and increased erythrocyte levels of phosphatidylcholine hydroperoxide (PCOOH) and plasma metHb. Antioxidants treatment significantly palliated single HD course-induced oxidative stress, plasma and RBC PCOOH, and plasma metHb levels, and preserved erythrocyte metHb /ferricyanide reductase activity in an order VC > ERW > VE-coated dialyzer. However, ERW had no side effects of oxalate accumulation easily induced by VC. Six-month ERW treatment increased hematocrit and attenuated proinflammatory cytokines profile in the HD patients. In conclusion, ERW treatment administration is effective in palliating HD-evoked oxidative stress, as indicated by lipid peroxidation, hemolysis, and overexpression of proinflammatory cytokines in HD patients.

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In patients undergoing hemodialysis (HD), the interaction of blood with non-biological materials of the extracorporeal circuit can activate polymorphonuclear leukocytes to produce quantities of reactive oxygen species (ROS), which impair neighboring tissues/cells (including RBCs) and evoke an inflammatory response.[1–4] The plasma levels of proinflammatory cytokines (e.g., interleukin (IL)-1, IL-6, tumor necrosis factor-α, and C-reactive protein) are significantly elevated in uremic patients on dialysis,[3,5] and the expression can be augmented further even after a single HD session.[3,6] Increased oxidative stress and proinflammatory cytokines in HD patients are related to malnutrition,[7] resistance to erythropoietin therapy,[8] cardiovascular events,[9,10] and high mortality.[11]

There is increasing evidence that oxidative stress plays a key role in the genesis and severity of dialysis anemia.[12–14] It reduces RBC survival,[15] impairs the effect of erythropoietin,[16,17] and increases the susceptibility to hemolysis owing to inflammatory, infectious, and mechanical stimuli.[13,18] During the process of oxidative stress, RBCs are subject to membrane lipid peroxidation and susceptible to destruction.[19–21] Increased ROS can oxidize oxyhemoglobin to yield H_2O_2 and methemoglobin (metHb)[22,23] that, in turn, leads to tissue hypoxia,[24] endothelial cell G_2/M arrest, and apoptosis.[25] Increased H_2O_2 repressed metHb reductase expression in HeLa cells;[26] deficiency of the enzyme, an autosomal recessive trait, results in hereditary methemoglobinemia (hemolysis).[27] A *trans*-plasma membrane electron transport system (e.g., nicotinamide adenine dinucleotide (reduced form)-ferricyanide reductase and NADH-metHb reductase) is present on RBC membranes and plays a role to reduce cytotoxic ferricyanide/metHb to functional ferrocyanide/oxyhemoglobin.[28,29] Intravenous vitamin C (VC)- or vitamin E (VE)-coated dialyzer can improve HD-enhanced erythro-

cyte lipid peroxidation and hemolysis via the preservation of NADH-ferricyanide reductase and NADH-metHb reductase.[4]

Electrolyte-reduced water (ERW) obtained by electrolysis scavenges $O_2^{-\bullet}$, H_2O_2, and HOCl,[3,30] and protects DNA from oxidative damage.[30] 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.[30] Active hydrogen in ERW may be an ideal scavenger against ROS because it does not produce oxidized molecules after reduction like other organic antioxidants (VC, VE, and polyphenols).[31] Our previous data reported that ERW administration diminished HD-enhanced H_2O_2 and HOCl activity, minimized atherosclerotic, oxidized and inflammatory markers, and partly restored total antioxidant status (TAS) during 1-month treatment.[3] In this study, 6 monthd of HD session was used in this study as *in vivo* system to evaluate the effects of ERW on dialysis-induced oxidative stress as indicated by erythrocyte lipid peroxidation, erythrocyte reductase activity, and metHb (hemolysis) levels. We also examined the long-term outcome of ERW on hematocrit, erythropoietin dose, and proinflammatory cytokine profiles in patients with end-stage renal disease (ESRD).

RESULTS

ERW-reduced HD-enhanced ROS formation in the blood

An average 15-fold increase in blood ROS activity was noted in patients who underwent HD without ERW treatment. Replacement of ERW could effectively suppress the ROS formation by $75 \pm 14\%$ in HD patients (Figure 1a). VC- or VE-coated dialyzer inhibited HD-enhanced ROS by 83 ± 11 or $49 \pm 9\%$.

Post-HD blood samples obtained from the group were used for examining the effects of several antioxidants. The augmented blood ROS counts were greatly inhibited by an H_2O_2 scavenger, catalase (by $68 \pm 8\%$), and partially depressed by superoxide dismutase ($14 \pm 5\%$) and epigallocatechin-3-gallate ($16 \pm 5\%$) (Figure 1b), indicating that the majority of the blood ROS activity was derived from H_2O_2. ERW depressed ROS activity by $60 \pm 8\%$.

ERW preserved HD-reduced plasma TAS

In the XERW group, the post-HD plasma VC and TAS levels were significantly decreased (Figure 2). This indicates that HD can cause acute oxidative stress and acute loss of plasma VC. In the VC group, intravenous VC significantly ($P < 0.05$) increased the plasma VC and TAS levels in ESRD patients receiving HD. Patients for whom the VE-coated dialyzer was used did not seem to have restored plasma VC and TAS levels. ERW treatment could restore plasma VC and TAS levels. In all four groups, there was no significant change in plasma VE and oxalate concentrations. However, an increased tendency of oxalate concentration was found in the VC group, suggesting a possibly adverse side effect of long-term intravenous VC.

ERW decreased HD-enhanced ROS and PCOOH in the plasma and RBC membrane

In our previous studies,[3,4] a single session of HD did not affect the levels of lipid profiles in the ESRD patients. The baseline level of pre-HD plasma phosphatidylcholine hydroperoxide (PCOOH) and erythrocyte membrane PCOOH was similar among the four groups of patients. However, in the XERW group, the post-HD plasma PCOOH (205 ± 24 pmol/ml) and post-HD erythrocyte membrane PCOOH levels (310 ± 39 pmol/ml) were significantly ($P < 0.05$) increased after HD (Figure 3). Intravenous VC significantly ($P < 0.05$) prevented increases in post-HD plasma PCOOH (110 ± 15 pmol/ml) and in post-HD RBC membrane PCOOH levels (168 ± 25 pmol/ml). In the VE group, the VE-coated dialyzer also decreased the increase in post-HD plasma PCOOH level (145 ± 21 pmol/ml, $P < 0.05$). On the other hand, the VE-coated dialyzer also prevented the increase in post-HD RBC membrane PCOOH levels (178 ± 29 pmol/ml, $P < 0.05$). In the ERW group, ERW treatment significantly reduced post-

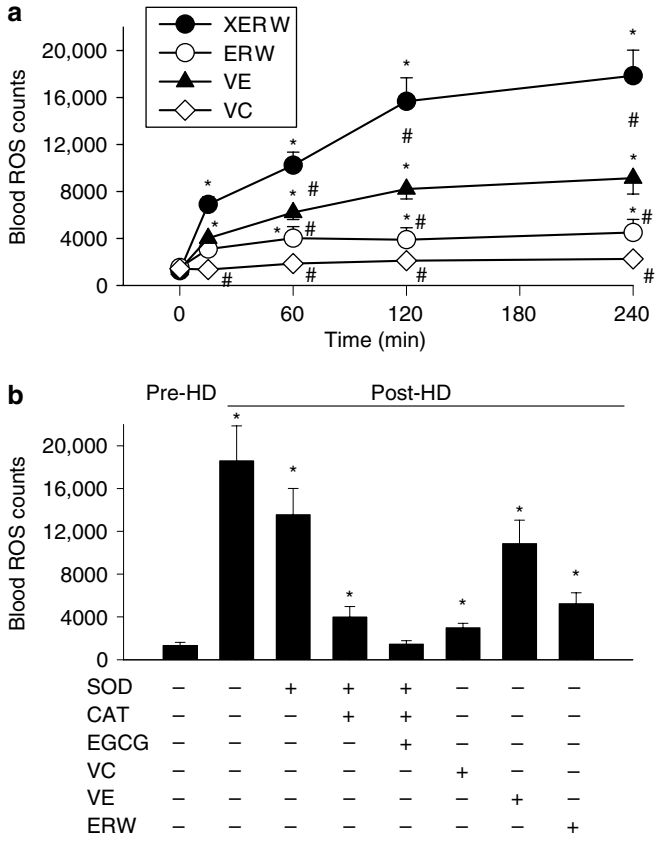

Figure 1 | (a) On palliating ROS formation in HD-activated blood, the effects of intravenous VC, a VE-coated dialyzer, and ERW are clarified. (**b**) The effects of superoxide dismutase, catalase (CAT), epigallocatechin-3-gallate (EGCG), VC, VE, or ERW on post-HD-enhanced blood ROS activity *in vitro* are displayed for comparison. VC group ($n = 15$), HD patients with intravenous VC; VE group ($n = 15$), HD patients with VE-coated dialyzer; ERW group ($n = 15$), HD patients with ERW treatment; and XERW group ($n = 15$), HD patients without VC, VE-coated dialyzer, or ERW treatment. Pre-HD = before an HD session; post-HD = after the HD session (corrected for hemoconcentration). *$P < 0.05$ vs pre-HD value; #$P < 0.05$ vs XERW group.

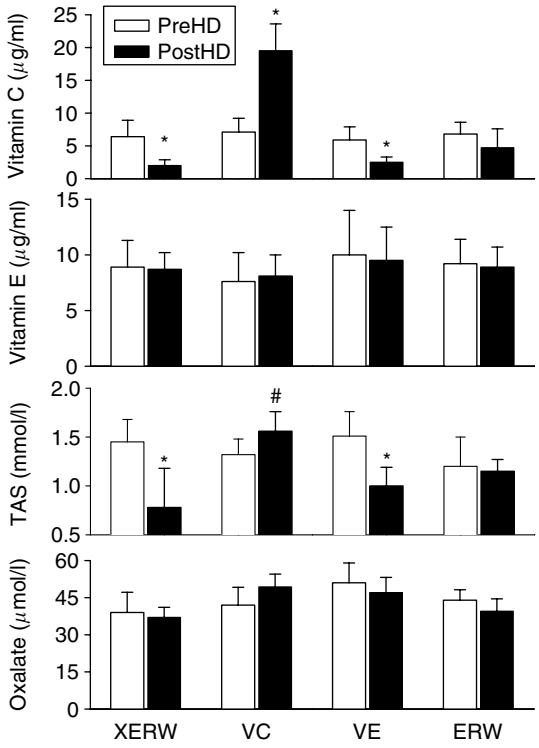

Figure 2 | Effects of intravenous infusion of VC, VE-coated dialyzer VE, or ERW on plasma VC, VE, TAS, and plasma oxalate (Oxalate) levels during a hemodialysis session. VC group ($n=15$), HD patients with intravenous VC; VE group ($n=15$), HD patients with VE-coated dialyzer; ERW group ($n=15$), HD patients with ERW dialysate; and XERW group ($n=15$), HD patients without VC, VE-coated dialyzer, or ERW dialysate. Pre-HD = before an HD session; post-HD = after the HD session (corrected for hemoconcentration). $^{*}P<0.05$ vs pre-HD value; $^{\#}P<0.05$ vs XERW group.

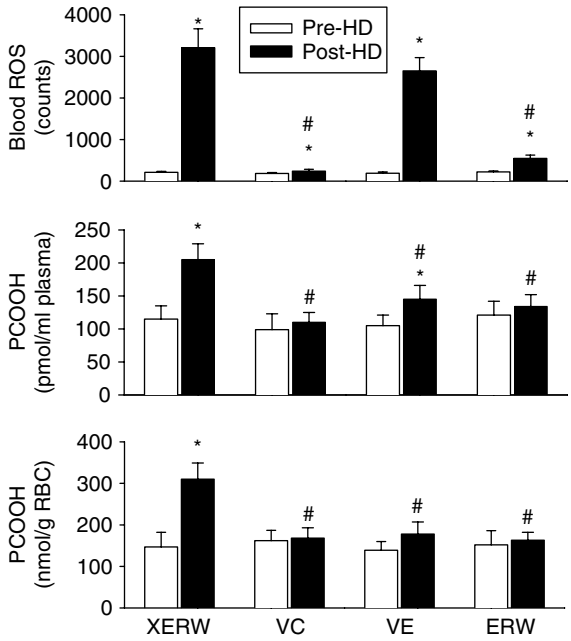

Figure 3 | Mean values of pre/post-HD blood H_2O_2-luminol counts, and PCOOH level in plasma and in RBC membrane are displayed. VC group ($n=15$), HD patients with intravenous VC; VE group ($n=15$), HD patients with VE-coated dialyzer; ERW group ($n=15$), HD patients with ERW dialysate; and XERW group ($n=15$), HD patients without VC, VE-coated dialyzer, or ERW treatment. Pre-HD = before an HD session; post-HD = after the HD session (corrected for hemoconcentration). $^{*}P<0.05$ vs pre-HD value; $^{\#}P<0.05$ vs XERW group.

HD plasma PCOOH (134 ± 18 pmol/ml, $P<0.05$) and post-HD RBC membrane PCOOH levels (163 ± 19 pmol/ml, $P<0.05$).

ERW reduced HD-triggered metHb formation

A single session of HD inhibited the activities of NADH-metHb reductase and NADH-ferricyanide reductase in erythrocytes, and it increased plasma metHb levels (Figure 4). Intravenous VC, VE, or ERW treatment significantly preserved the activities of erythrocyte ferricyanide reductase and erythrocyte metHb reductase, and decreased the HD-augmented metHb level. These findings imply that ERW treatment was effective in preventing oxidative stress on erythrocytes.

Long-term effect of ERW on HD-induced oxidative stress and inflammatory markers

To determine the long-term effects of ERW in the ESRD patients undergoing chronic HD, we recorded the post-HD blood ROS, post-HD levels of activities of NADH-metHb reductase and NADH-ferricyanide reductase in erythrocyte membrane, and post-HD plasma metHb level in the ERW- and XERW-treated patients for 6 months. As shown in Figure 5, after 6 months of ERW treatment in the ESRD patients with chronic HD, the increased post-HD blood ROS was decreased ($P<0.05$) when compared to XERW group, whereas the post-HD levels of activities of NADH-metHb reductase and NADH-ferricyanide reductase were increased ($P<0.05$) when compared to the XERW group. The post-HD plasma metHb level was also significantly decreased ($P<0.05$) in the ERW group when compared to the XERW group. The long-term outcome showed that 6-month ERW treatment significantly increased hematocrit (from 28.9 ± 0.7 to $31.5\pm0.6\%$) at a similar dose of erythropoietin administration in the 43 chronic HD patients (Figure 6). However, the differences of hematocrit change were not statistically significant in the XERW group (from 31.0 ± 1.0 to $30.7\pm0.4\%$) even at a similar dose of erythropoietin. Furthermore, after 6 months of ERW treatment, 26 plasma cytokines critical to the inflammatory response are also downregulated (Figure 7).

DISCUSSION

Our study indicates that HD activated polymorphonuclear leukocytes and monocytes to release of ROS, mostly H_2O_2[3,4] and was accompanied by adverse events in ESRD patients, including (1) increased amount of ROS in the blood; (2) peroxidation of plasma lipid and RBC membrane lipid;

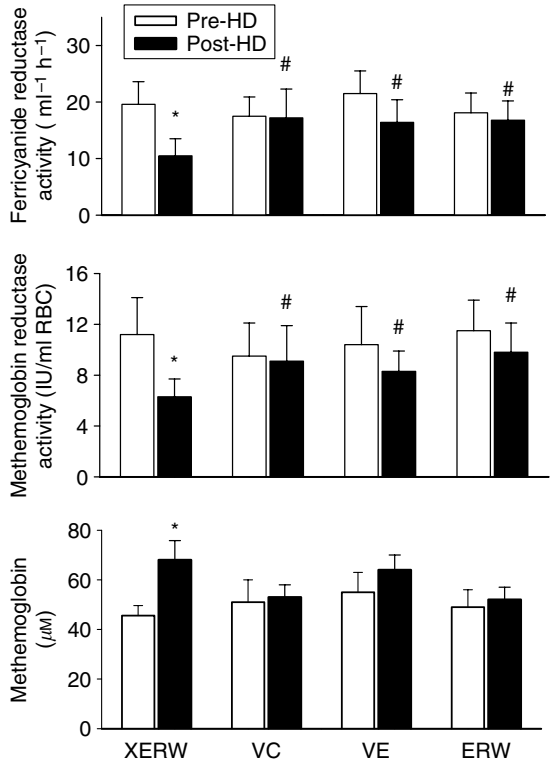

Figure 4 | Effects of intravenous infusion of VC, VE-coated dialyzer, or ERW treatment on HD-affected erythrocyte ferricyanide reductase activity, metHb reductase activity, and plasma metHb level. VC group ($n = 15$), HD patients with intravenous VC; VE group ($n = 15$), HD patients with VE-coated dialyzer; ERW group ($n = 15$), HD patients with ERW dialysate treatment; and XERW group ($n = 15$), HD patients without VC, VE-coated dialyzer, or ERW treatment. Pre-HD = before an HD session; post-HD = after the HD session (corrected for hemoconcentration). $^{*}P < 0.05$ vs pre-HD value; $^{\#}P < 0.05$ vs XERW group.

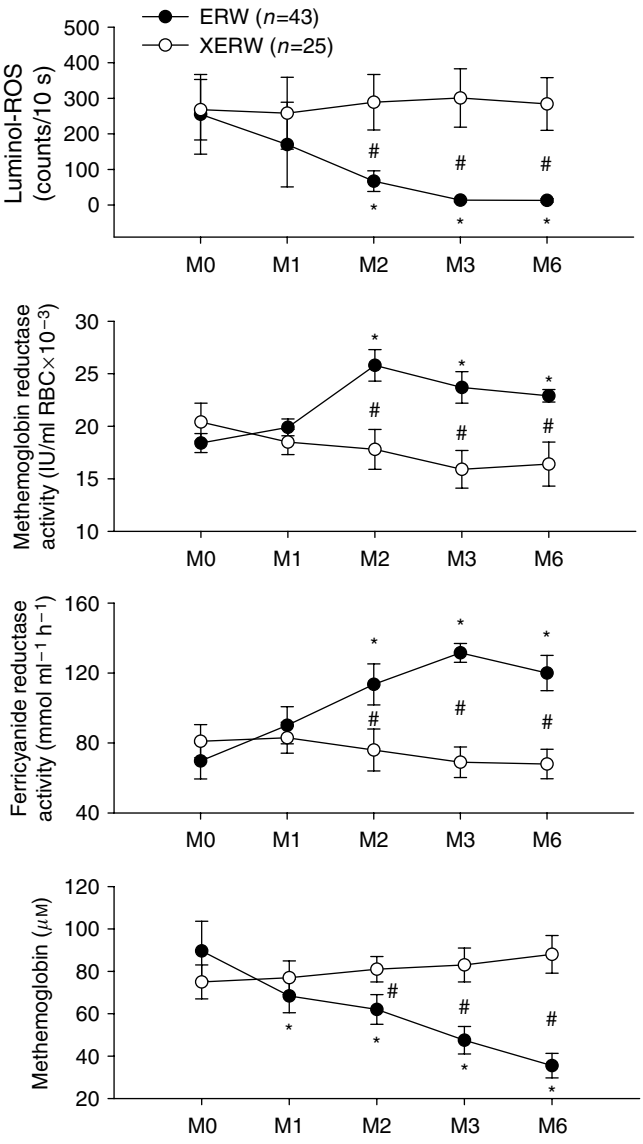

Figure 5 | Effects of 6-month ERW treatment on post-HD-affected erythrocyte ferricyanide reductase activity, metHb reductase activity, and plasma metHb level in 43 ESRD patients. Twenty-five ESRD patients without ERW treatment (XERW) were used as control group. M0, before ERW treatment; M1, ERW treatment for 1 month; M2, ERW treatment for 2 months; M3, ERW treatment for 3 months; and M6, ERW treatment for 6 months. $^{*}P < 0.05$ vs M0 value.

(3) inhibition of the activity of erythrocyte reductases, leading to hemolysis. The application of ERW can decrease HD-enhanced blood ROS production, RBC lipid peroxidation, and hemolysis via the preservation of NADH-ferricyanide reductase and -metHb reductase activity. This beneficial long-term outcome of 6-month ERW treatment can improve the hematocrit without the increment of erythropoietin dosage.

The oxidative stress associated with uremia is exacerbated by hemodialysis since neutrophils and monocytes, activated by contact with the dialysis membranes, release large amounts of ROS.[1–4] The deleterious effects of ROS on carbohydrates, lipids, and proteins have a pathological role in many inflammatory diseases, most of which are frequent in HD patients.[5,12] In particular, the oxidation of polyunsaturated fatty acids on the RBC membrane[3,4,12] increases RBC rigidity and reduces their deformability,[32,33] leading to greater susceptibility to hemolysis,[4] and shorter survival.[15] ROS may cause subsequent oxidation and release of other oxidized metabolites and are increased after HD session.[3,4] As a primary peroxidation product from membrane phospholipids, the level of PCOOH in the plasma and erythrocyte membrane was increased by HD.[3,4] Further, the significant increases of metHb and the decreased activity of two RBC reductases (NADH-ferricyanide reductase and -metHb reductase) in post-HD plasma and RBC implicate potential RBC damage secondary to lipid peroxidation. Human oxyhemoglobin reacts with $Fe(II)(CN)_5H_2O^{3-}$ to yield H_2O_2 and metHb.[22] Further, metHb production can be accelerated by H_2O_2.[34] In our previous study, HD-triggered H_2O_2 formation was positively correlated with the degree of hemolysis and with metHb levels.[4]

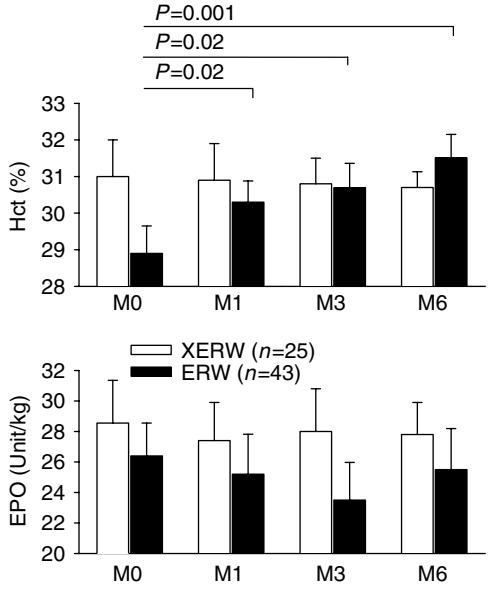

Figure 6 | Effects of 6-month ERW treatment on post-HD-affected hematocrit (Hct) and erythropoietin dose (EPO) in 43 ESRD patients. Twenty-five ESRD patients without ERW treatment (XERW) were used control group. M0, before ERW treatment; M1, ERW treatment for 1 month; M3, ERW treatment for 3 months; and M6, ERW treatment for 6 months.

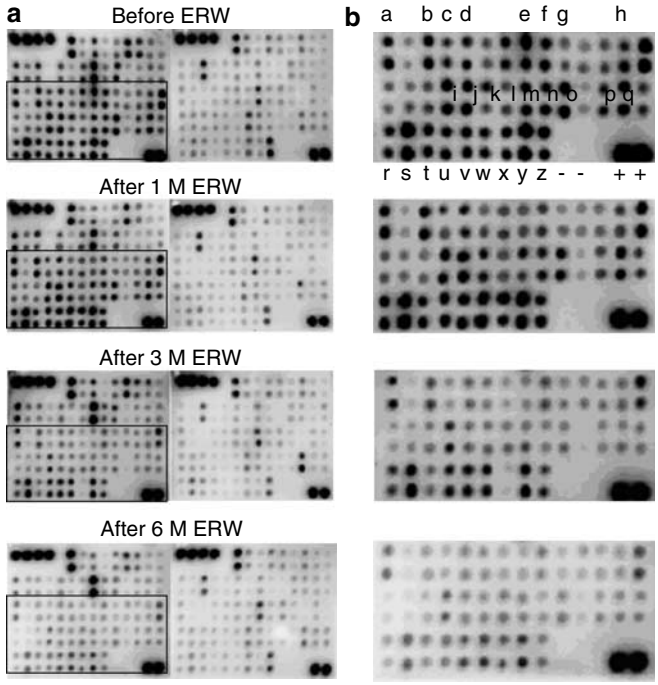

Figure 7 | Determination of multiple cytokines determination by cytokine antibody array in the pre-HD plasma of one ESRD patient before and during 6-month ERW treatment. (**a**) The pre-HD plasma cytokine profiles and (**b**) amplified dot plot of cytokines before, after 1-month (1 M), 3-month (3 M), and 6-month (6 M) ERW treatment are displayed. Note that ERW treatment significantly reduced the dot-spot intensity of proinflammatory cytokines (a–z) after 6 M of ERW treatment. a, insulin growth factor-1; b, IL-13; c, IL-15; d, IL-16; e, IL-1β; f, IL-1ra; g, IL-2; h, IL-6; l, membrane cofactor protein-2; j, membrane cofactor protein-3; k, membrane cofactor protein-4; l, macrophage-colony-stimulating factor; m, MDC; n, MIG; o, macrophage inflammatory protein-1δ; p, NAP-2; q, NT-3; r, platelet-derived growth factor-BB; s, RANTES; t, stem cell factor; u, SDF-1; v, TARC; w, tumor growth factor-β1; x, tumor growth factor-β3; y, tumor necrosis factor-α; z, tumor necrosis factor-β; −, negative dot; +, positive dot.

Based upon the interesting clinical improvement of a variety of diseases by intake of reduced water since 1985, Hayashi[35] proposed the hypothesis 'Water Regulating Theory'. The ideal scavenger for ROS should be active atomic hydrogen. Active atomic hydrogen can be produced in ERW near the cathode during electrolysis of water. ERW exhibits high pH, low dissolved oxygen, extremely high dissolved molecular hydrogen, and extremely negative redox potential values.[30] Shirahata *et al.*[30] suggest that the superoxide dismutase- and catalase-like activity of ERW is not owing to the dissolved molecular hydrogen but owing to the active atomic hydrogen with a higher reducing ability that may participate in ROS-scavenging activity. Happe *et al.*[36] indicate that hydrogenases, which are among in the oldest enzymes (3.8 billio years old), can reversibly split molecular hydrogen to produce active atomic hydrogen, which participates in the redox regulation of cellular 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.[30] Active hydrogen in ERW may also be an ideal scavenger against ROS because it does not produce oxidized molecules after reduction like other organic antioxidants (VC, VE, and polyphenols).[3,31] ERW, VC and catechins completely scavenged $O_2^{-\bullet}$ produced by the hypoxanthine-xanthine oxidase system in sodium phosphate buffer.[30] Furthermore, ERW, catalase and VC could directly scavenge H_2O_2.[3,30] We found that ERW and epigallocatechin-3-gallate can scavenge both H_2O_2 and HOCl, but VC only availably 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 previous[3] and the present experiment. Furthermore, in spite of the presence of ERW, our previous report showed a significant increase in plasma PCOOH and a decrease in TAS from pre- to post-dialysis in the ESRD patients, whereas the present study showed no difference in PCOOH and TAS from pre- to post-dialysis in the ESRD patients. This discrepancy may be due to a diet control in the present study, because a dietary factor increased plasma triglyceride and very low-density lipoprotein levels, which could lead to an increase in PCOOH level and a reduction in TAS from pre- to post-dialysis even in the presence of ERW in our previous study.[3] This result indicates that ERW with a higher reducing ability and/or direct ROS-scavenging activity could be used in HD patients by its stable characteristics, cheaper expense, and strong antioxidant activity to relieve

HD-evoked oxidative stress, preserve the activity of erythrocyte reductases and, and increase hematocrit during six months of treatment. Furthermore, it is well known that VC overdoses or long-term usage may cause secondary oxalosis via an increase in plasma oxalate levels and the deposition of calcium oxalate in various tissues.[4,37] Therefore, the application of ERW treatment could prevent the increased plasma oxalate level during a single HD session or after six-month treatment.

ESRD patients on maintenance HD show systemic inflammation due to uremia and dialysis and suffer from cardiovascular events[7–11] and severe anemia.[38] HD-augmented hemolysis has been proposed as a significant factor in regard to HD-related shortened erythrocyte survival.[28] VE supplementation for HD patients resulted in reduced erythropoietin dosage requirements.[16] HD patients with stable hemoglobin levels and not receiving erythropoietin therapy exhibited a higher OH-scavenging activity when compared with those in need of erythropoietin:[39] it may be argued that erythropoietin or anemia-associated increments in the oxidant burden may be counterbalanced by a higher consumption of antioxidants like VC. Tarng *et al.*[40] reported that the dose of erythropoietin as well as the ratio of erythropoietin/hemoglobin was found to correlate positively with serum concentrations of 8-hydroxy-2′-deoxyguanosine, an oxidation product of DNA. VC influenced renal synthesis of erythropoietin in the kidneys, and production of erythropoietin was enhanced in the presence of antioxidative cocktail comprising vitamins A, E, and C. Also, pro-oxidants reduced erythropoietin synthesis in human hepatoma cells.[41] Our data showed that 6 months of antioxidant ERW treatment significantly increased hematocrit but did not increase erythropoietin dose in the 43 ESRD patients. Antioxidants like VE and anti-inflammatory drugs can attenuate chronic HD-evoked cardiovascular events and neutrophil and endothelial cell apoptosis.[41–44] The present results showed that 26 proinflammatory cytokines are downregulated after 6 months of ERW treatment. These findings provide new insight into the molecular events that resolve anemia and inflammation in chronic HD patients by the ERW treatment. A further study for clarification of ERW on immunity needs to be determined.

In conclusion, our findings demonstrate that ERW treatment is effective in palliating HD-evoked oxidative stress, as indicated by hemolysis and lipid peroxidation, in HD patients. Long-term benefits of this novel therapy are associated with an increase of hematocrit level, improvement of erythropoietin responsiveness, and downregulation of proinflammatory cytokines in chronic HD patients.

MATERIALS AND METHODS

Patients

To assess the extent of oxidative stress in blood of ESRD patients, 25 patients (14 men and 11 women) without any treatment (XERW group), 15 patients (seven men and eight women) with intravenous VC group (1 g in 250 ml of saline infused over 4 h of HD), 15 patients (eight men and seven women) with VE-coated dialyzer (VE group), 43 patients (28 men and 15 women) subjected to ERW treatment (ERW group) with 10.8 ± 0.5 mg/dl of creatinine, subjected to maintenance HD at Wan-Hwa Hospital for a period of 12 ± 2 months after informed consent obtained were enrolled in the ERW study. The criteria for patient selection included the absence of habit of smoking, malignancy, inflammatory disorders, chronic or acute infections, supplementation of VC or VE, and treatment either oral or intravenous iron, or anti-inflammatory drugs 3 months before enrollment. The mean age was 58 ± 3 years old (mean $\pm$ s.e.m., range 42–82 years). As the subjects for 6-month study were recruited into the ERW and XERW groups, we found that at entry the study the XERW group had a high, but not significant, baseline hematocrit level than the ERW group (31.0 ± 1.0 vs 28.9 ± 0.7%, $P = 0.097$). A similar level in blood urea nitrogen (63.5 ± 2.7 vs 71.4 ± 3.3 mg/dl, $P = 0.07$), creatinine (10.0 ± 0.35 vs 11.0 ± 0.51 mg/dl, $P = 0.10$), albumin (3.82 ± 0.05 vs 3.78 ± 0.07 g/dl, $P = 0.57$), and ferritin (395 ± 20 vs 373 ± 38 ng/ml, $P = 0.596$) was noted in the ERW and XERW groups. All HD patients except VE group used hemodialyzer (AM-Bio-HX-90 and AM-Bio-HX-100 biomembrane: Asahi Kasei Med Co., Tokyo, Japan). A VE-coated EE18 Excerbrane® membrane (Terumoc Co., Japan) was used in the VE group. The VC solution (Tai-Yu Pharmaceutical, Taipei, Taiwan) was prepared in 1 g/250 ml of saline before use. The blood flow rate during the dialysis sessions was 250–300 ml/min and dialysate flow 500 ml/min. The surface area of these two dialyzers was 1.5 and 1.8 m^2, respectively, and patient treatment with either membrane lasted for 6 montha. The kt/v of all these patients is maintained between 1.3 and 1.6. The cause of ESRD was diabetes in 55 patients and chronic glomerulonephritis in 43 patients. HD 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 and any antioxidants such as VC or VE during 6 months of tested period. The permission of clinical trial was approved by the meeting of Human Research Committee of the Wan-Hwa Regional Hospital.

ERW system

HD-24K (Nihom Trim Co., Osaka, Japan) was structured as described in detail previously.[3] In brief, raw water was supplied after compressed by the compression pump and supplied into a compartment for electrolysis through a solanoid valve, which opens and closes synchronizing with the signal of water level sensor in electrolyzed water storage tank and flow sensor. At the electric compartment, the supplied raw water was electrolyzed by direct current supplied to + and − electrode plate across diaphragm and at the side of − electrode plate reduced water containing active hydrogen and lower redox potential value. The series of operation are automatically carried out according to the level of reduced water tank. 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 reduced water produced by HD-24K was collected in reduced water tank, and then was supplied to usual raw water processing system for HD by compress with compressing pump. The intensity of electrolysis of HD-24K is adjusted to 'level 2' and the ERW that indicates pH 9.8 right after taking from 'out' of HD-24K is used in this study. The quality of ERW for HD was approved by Yen Tjing Ling Industrial Research Institute National Taiwan University with the No. E89A299D277, which is required by the American Association of Medical Instruments (AAMI). The ERW dialysates maintain the properties

of lower redox potential value when compared to that of the non-ERW dialysate (150±5 296±5 mV). The microbiological quality of the final dialysates meets the AAMI standard (<bacteria of 200 colony-forming units/ml and <endotoxin concentration of 2 EU/ml). Because culture results do not become available for at least 48 h, during which time bacteria continue to proliferate, the final dialysate action level also meet AAMI standard (<bacteria and endotoxin of 50 colony-forming units//ml and 1 EU/ml, respectively). The endotoxin level in the water used for HD is 0.061±0.003 EU/ml measured by an endotoxin 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

Blood (10 ml) was collected via the arterial line of the HD circuit, before the start and the end of an HD session, into a heparinized sterile test tube and was processed within 2 h. Concerning the effects of hemoconcentration during HD, the post-HD levels of various plasma biochemicals were corrected with the following equation:

$$\text{Corrected post} - \text{HD value} = \text{measured post} - \text{HD value} \times (C_{\text{pre-HD}}/C_{\text{post-HD}})$$

The whole-blood cell count, including the hemoglobin level and hematocrit, were determined with an autoanalyzer (Coulter STKS, Hialeah, Finland). Plasma levels of urea nitrogen and creatinine were determined with an autoanalyzer (Hitachi 736–15, Ibaraki, Japan). The plasma oxalate concentration was determined by an enzymatic method.[37]

Measurement of ROS activity in whole blood and plasma

To determine the ROS activity in whole blood and plasma, we sampled and isolated plasma from the four groups of patients at various time points through the first HD session of the study. After sampling, 0.2 ml of whole blood or plasma was immediately added to the luminol solution, and ROS signals were measured as described previously.[4]

To identify the types of ROS in the HD-activated blood, we studied the effects of various ROS inhibitors, including superoxide dismutase (an $O_2^{-\bullet}$ scavenger, 30 U), catalase (an H_2O_2 scavenger, 30 U), and epigallocatechin-3-gallate (an HOCl and H_2O_2 scavenger, 10 μg).[4] To test the *in vitro* effects of ERW, we also compared the ROS signals in blood samples obtained from the N group before and after treatment with ERW.

Plasma VC and VE assay

To preserve VC, an aliquot of plasma was deproteinized with 10% metaphosphoric acid (1:1, vol/vol), and the supernatant was kept at −80°C. Plasma VC was measured by high-performance liquid chromatography (Hitachi D-7000 HPLC system) with ultraviolet detector at 254 nm with a Cosmosil 5 C_{18}-MS column (250 × 4.6 mm ID) with a 5-μm particle size.[45]

VE was measured, after extraction with ethanol and hexane, by high-performance liquid chromatography with UV detection at 292 nm[46] with a Cosmosil 5C_{18}-MS column (150 × 4.6 mm ID) with a 5-μm particle size. The system manager software (D-7000, version 3.0; Hitachi) was used for VC and VE peak integration and data acquisition.

The extraction recoveries of standards recoveries of VC and VE within the concentration range of the assay were 85 and 96%. For VC and VE, within-day variability was lower than 13.5%, whereas between-day variability did not exceed 14.8%. Within-day variability averaged 11.4±2.5% for VC and 6.1±1.6% for VE. Mean between-day variability was 11.9±2.7% for VC and 6.3±1.5% for VE.

Measurements of plasma TAS

We measured the TAS of plasma (20 μl) by a commercial kit (TAS kit, Randox, San Franciso, CA, USA) according to the manufacturer's instructions.[3,4]

Measurement of lipid peroxidation products

A lipid peroxidation product, PCOOH, originating from oxidized phosphatidylcholine is a primary lipid peroxidation product,[47] which was measured to serve as an indication of oxidative stress.[3,4] The amounts of PCOOH in plasma and erythrocytes were determined in duplicate by chemiluminescence high-performance liquid chromatography (CL-HPLC, Tohoku Electronic Ind. Co., Sendai, Japan).

Spectrophotometric assessment of oxidized hemoglobin and RBC reductase activities

HD-induced oxidative stress, via erythrocyte membrane lipid peroxidation, would lead to erythrocyte destruction and hemoglobin release. We determined the plasma concentration of metHb spectrophotometrically, as described previously.[34] The hemolysis score was determined by spectrophotometric measurement at 540 nm, and with a Coulter counter (Coulter Onix, Miami, FL, USA). A reduced activity of RBC *trans*-plasma membrane electron transport system, including ferricyanide reductase and metHb reductase, could lead to cell damage and increase plasma metHb and H_2O_2 concentration.[28,29] RBC ferricyanide reductase activity was measured as described previously.[48] metHb reductase activity was assayed by use of 2,6-dichlorophenol indophenol as an electron acceptor.[49]

Statistical analysis

All values are expressed as mean±s.e.m. Within-group comparisons between pre- and post-HD values were performed by paired *t*-test. For longitudinal data, repeated measures analysis of variance with *post hoc* analysis with the Bonferroni procedure were used to analyze the between- and within-group differences. $P<0.05$ was adopted as indicating statistical significance.

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