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*Original Article*

# Electrolysed-reduced water dialysate improves T-cell damage in end-stage renal disease patients with chronic haemodialysis

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## Abstract

**Background.** T-cell damage by increased oxidative stress in end-stage renal disease (ESRD) patients undergoing chronic haemodialysis (HD) led to the increased T-cell apoptosis and the alteration of surface markers and Th1/Th2 ratio in $CD4^+$ T lymphocytes. Antioxidant electrolysed-reduced water (ERW) was used as the dialysate in ESRD patients undergoing chronic HD to test for improved oxidative stress-related T-cell apoptosis, alterations of surface markers and intracellular cytokine profile.

**Methods.** We evaluated apoptosis formation by annexin V, CD25-related surface markers, and cytokine ratio of Th1/Th2 in $CD4^+$ T lymphocytes and Tc1/Tc2 in $CD8^+$ T lymphocytes of 42 ESRD patients haemodialysed with ERW for 1 year.

**Results.** In comparison to 12 healthy individuals, the ESRD patients had more T-cell apoptosis and less $CD3^+$, $CD4^+$ and $CD8^+$ T cells and CD25/CD69/CD94/$CD3^+$ phenotypes at baseline. Lower intracellular IL-2 and IFN-γ levels in the Th1/$CD4^+$ and Tc1/$CD8^+$ cells and higher intracellular IL-4, IL-6 and IL-10 levels in the Th2/$CD4^+$ and Tc2/$CD8^+$ cells were also noted in the ESRD patients. After a 1-year ERW treatment, the patients had a decrease in T-cell apoptosis and increases in $CD3^+$, $CD4^+$ and $CD8^+$ cell numbers and CD25/CD69/CD94/$CD3^+$ phenotypes in the T cells. The intracellular IL-2 and IFN-γ levels in the Th1/Tc1 cells significantly ($P < 0.05$) increased and the intracellular IL-4, IL-6 and IL-10 levels in the Th2/Tc2 cells decreased. Furthermore, the Th1/Th2 and Tc1/Tc2 cytokine ratios were improved toward a normal status.

**Conclusion.** One-year ERW treatment effectively ameliorated T-cell apoptosis, altered CD25-related surface markers and intracellular cytokine profile in the HD patients.

**Keywords:** apoptosis; electrolysed reduced water; end-stage renal disease; oxidative stress; T-lymphocyte

## Introduction

In end-stage renal disease (ESRD) patients undergoing chronic haemodialysis (HD), the activation of leukocytes with non-biological materials of the extracorporeal system can further produce burst amounts of reactive oxygen species (ROS), such as $O_2^{-\bullet}$, $H_2O_2$ and HOCl, which impair erythrocytes, immune cells and endothelial cells and subsequently lead to anaemia and resistance to erythropoietin therapy [1,2], cardiovascular diseases [3–5], malnutrition [6], inflammation [1,3,7,8], immunodeficiency [9–11] and high mortality [12]. Increased plasma levels of several proinflammatory cytokines (IL-1, IL-6 and TNF-α) and C-reactive protein also frequently occur in ESRD patients with HD [1,3,13]. The aggravated oxidative stress and proinflammatory cytokines in ESRD patients contribute to repeated infections, increased incidences of malignancy, impaired responses to vaccinations [14], impaired cell-mediated immunity and reduced $CD4^+$/$CD8^+$ T-lymphocyte ratios [9–11].

In dialysis and chronic renal failure patients, reduced T-cell counts and impaired T-cell proliferation have been reported [9]. Aberrant T-cell activation increases the pronicity of lymphocytes to undergo cell death via the CD95/CD95L(Fas/FasL)TNFR1 pathway in pre-dialysis and dialysis patients [10]. Increased oxidative stress leads to apoptotic programmed cell death [15]. $CD4^+$ T (Th) and $CD8^+$ T (Tc) lymphocytes can be functionally divided into Th1/Th2 and Tc1/Tc2 subsets based on the secretion level of IFN-γ (T1) or IL-4 (T2) [16,17]. An increased percentage of INF-$\gamma^+$ and IL-$4^+$ T cells has been found in chronic renal failure patients compared with healthy subjects [11]. Additionally, a significantly increased percentage of $CD4^+$ cells is associated with high Th1 cytokine production in HD patients [11]. On the other hand, the activation of the T-cell receptor/CD3 complex leads to the immediate

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activation of transcription factors that regulate a variety of activation-associated genes of cytokines and surface receptors for coordinating the immune response [18]. Activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB) translocation from the cytoplasm to the nucleus regulate transcriptional induction of several cytokines and T-cell activation-induced proteins [19–22]. Downregulation of AP-1 and NF-κB DNA-binding activity by antioxidants inhibits human T lymphocytes activation and inflammation-based cardiovascular diseases [23,24].

Electrolysed-reduced water (ERW) with rich atomic hydrogen obtained by electrolysis scavenges $O_2^{-\bullet}$, $H_2O_2$ and HOCl and protects DNA, lipids and proteins from oxidative damage [1,3,25]. In addition, it does not produce oxidized molecules after reduction like other organic antioxidants [1,3,26]. Our serial studies indicated that ERW dialysate diminished HD-enhanced $H_2O_2$ and HOCl activity, minimized atherosclerotic, oxidized and inflammatory markers and improved severity of anaemia during treatment [1,3]. Additionally, ERW reduced plasma levels of IL-6 and C-reactive protein [3]. A significant reduction in several proinflammatory cytokine levels as analysed by a cytokine array was indicated after 6 months of ERW treatment [1]. Thus, we speculated that a 12-month ERW treatment may have a protective potential on T cells by modulating T-cell apoptosis, altered CD25-related surface marker expression and intracellular Th1/Th2 and Tc1/Tc2 cytokine ratios in the ESRD patients with chronic HD.

## Materials and methods

### *Subjects*

All ESRD patients ($n$ = 55) in the dialysis centre of Wan-Hwa Regional Hospital underwent chronic HD with ERW dialysate during the same period. However, only 42 patients with informed consent met the following inclusion criteria: the absence of habitual smoking, malignancy, inflammatory disorders, chronic or acute infections, supplementation of vitamin C or E and treatment with either oral or intravenous iron or anti-inflammatory drugs in the preceding 3 months. The mean age of the enrolled patients was 57 ± 5 years (range 42 to 88 years). The causes of ESRD were type II diabetes mellitus in 15 patients and chronic glomerulonephritis in the other 27 patients. Of the 15 diabetic patients, 5 were treated with insulin injection, 6 with oral hypoglycaemic agents (OHA) and 4 with a combination of insulin and OHA. All 42 patients were on a normal diet without restriction of essential fatty acids. Their mean mid-week pre-dialysis creatinine level was 10.9 ± 0.5 mg/dL at baseline. During the dialysis sessions, the blood flow rate was 250–300 mL/min and the dialysate flow was 500 mL/min. One kind of haemodialyser with a surface area of 1.5 or 1.8 $m^2$ (AF-150 and AF-180 membrane type; Althane, Althin Medical Inc., USA) was adopted according to the individual's body weight. The Kt/V of all these patients was maintained between 1.3 and 1.6. We also enrolled 12 healthy controls (3 males and 9 females, 35 ± 6 years old). This clinical trial was approved by the Human Research Committee of Wan-Hwa Regional Hospital.

### *ERW system*

HD-24K (Nihom Trim Co., Osaka, Japan) was structured as described previously [3]. The quality of ERW for HD was approved by the Yen Tjing Ling Industrial Research Institute National Taiwan University with the no. E89A299D277 as required by the American Association of Medical Instruments (AAMI). The ERW dialysate maintained the properties of a lower redox potential value when compared to that of the non-ERW dialysate (150 ± 5 *vs* 296 ± 5 mV). The microbiological quality of the final dialysate met the AAMI standards: bacteria <200 colony-forming units/mL and an endotoxin concentration <2 EU/mL. The endotoxin level in the water used for HD was 0.050 ± 0.002 EU/mL as measured by an endotoxin ELISA kit (HBT-HIT301, Biocompare Inc., CA, USA) and an automated ELISA analyser (CODA, SN10373, CA, USA).

### *Measurements of cell death in T lymphocytes*

Ten millilitres of blood samples was collected from the healthy controls and the enrolled chronic HD patients just before the mid-week dialysis session. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation [10] for the analysis of T-cell apoptosis. Apoptotic and necrotic cell death in $CD3^+$ T lymphocytes was analysed in PBMCs from all ESRD patients by flow cytometry [10]. T cells were further assayed for apoptosis with FITC-labelled monoclonal antibody using the Annexin V kit (PharMingen, San Diego, CA, USA). Double labelling was performed at 37 °C as follows: propidium iodide (PI, 50 μg/mL) and Annexin V (2 μg/mL) were added to the culture medium for 2 h and then the staining was immediately analysed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

### *Surface marker expression*

$CD3^+$ T lymphocytes from whole blood were phenotyped by dual-colour flow cytometry using a standard procedure that included the establishment of a lymphocyte gate on a light scatter bit map and the use of the minimal acceptable antibody combinations, *i.e.* $CD3^+CD4^+$ and $CD3^+CD8^+$. Cells were adjusted to $0.5 \times 10^6$ cells/mL in phosphate buffer saline containing 10% foetal bovine serum (Hyclone, Logan, UT, USA). Native samples were incubated with fluorescence-labelled antibodies according to the manufacturer's instructions. Subsequently, 0.4 mL FACS-Flow® (BD Biosciences) was added. Lymphocytes were defined via forward scatter (FSC) and sideward scatter (SSC); their population showed a low SSC and an elongated width of FSC characteristics. To obtain mainly morphologically intact cells and to avoid degraded cells and debris, this population was gated in the more upper part of the FSC distribution and checked for an optimum of cell viability via PI dye exclusion. T-lymphocyte subpopulations were identified on the respective bivariate plots.

Phorbol myristate acetate (5 ng/ml) (PMA; Sigma Chemical Co., St. Louis, MO, USA) and ionomycin (500 ng/mL) (Sigma Chemical Co., St. Louis, MO, USA) were applied to the lymphocytes and incubated for either 4 or 24 h to measure surface marker expression and intracellular cytokine production in the activated T cells.

For the detection of CD25, CD69 and CD94, 0.25 mg/mL of FITC-conjugated antihuman CD25 (PharMingen), FITC-conjugated antihuman CD69 (PharMingen) and FITC-conjugated antihuman CD94 (PharMingen) were added to tubes (12 × 75 mm) containing the PMA and ionomycin-stimulated $CD3^+$ lymphocytes (50 μL of $10^7$/mL) designated for the measurement of activation and the samples were incubated in the dark at room temperature for 30 min. We used CD3-FITC for T-cell identification, CD3-FITC/CD4 conjugated to phycoerythrin (PE) for Th cell identification, CD3-FITC/CD8–phycoerythrin cyanin 5 for T-cell recognition and CD25 and CD69 for T-cell activation. The samples were then analysed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest software (Becton Dickinson).

### *Intracellular cytokine profile*

Flow cytometric evaluation of the intracellular expression of IFN-γ, IL-2, IL-4, IL-6 and IL-10 in the Th1/Th2 and Tc1/Tc2 cells was performed according to the manufacturer's instructions (Becton Dickinson, Application Note: Detecting Intracellular Cytokines in Activated Lymphocytes). Briefly, whole blood was collected into tubes containing sodium heparin and diluted in RPMI 1640 and was assayed within 6 h after collection. All activation procedures were performed in capped polystyrene test tubes. $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin and incubated for 4 h at 37 °C; brefeldin A (10 μg/ml) was added for the last 3 h of incubation. PMA and ionomycin are pharmacological T cell-activating agents and have the advantage of stimulating T cells of any antigen specificity. Brefeldin A is used to block intracellular transport mechanisms, thereby leading to an accumulation of cytokines in the cells.

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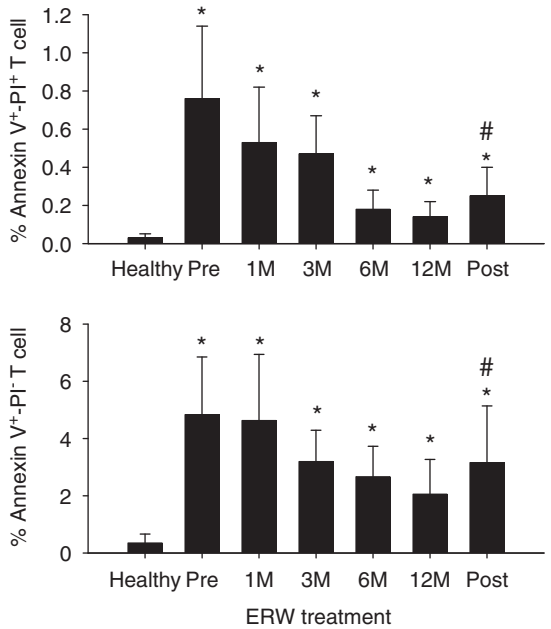


**Fig. 1.** ERW on T lymphocyte apoptosis. The effect of ERW dialysate treatment on % annexin $V^+$–$PI^+$ and annexin $V^-$–$PI^+$ in the T lymphocytes before ERW treatment (Pre), during the first month (1M), third month (3M), sixth month (6M) and 12th month (12M) of ERW treatment and 6 months post-ERW treatment (Post) was demonstrated. ERW treatment during 3–12 months significantly reduced % annexin $V^+$–$PI^+$ and % annexin $V^-$–$PI^+$ in the T lymphocytes of the 42 patients. An increase in % annexin $V^+$–$PI^+$ and % annexin $V^-$–$PI^+$ was found at 6 months post-ERW treatment. Mean ± SD. *P < 0.05 when compared to healthy controls ($n$ = 12); #P < 0.05 Post *vs* 12 months of ERW treatment.

T cells were stained with PE-conjugated anti-CD4 or anti-CD8 monoclonal antibodies at room temperature in the dark for 15 min. Intracellular staining was performed for 30 min at room temperature using FITC-conjugated anti-IFN-γ, FITC-conjugated anti-IL-2, FITC-conjugated anti-IL-4, FITC-conjugated anti-IL-6 or FITC-conjugated anti-IL-10 (PharMingen). CellQuest software and a FACSCalibur (BD Biosciences) were used for data collection and analysis.

*Statistical analysis*

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

## Results

The haematocrit levels of the patients were 30.3 ± 3.6% at Month 0, 29.6 ± 3.2% at Month 1, 30.3 ± 3.5% at Month 3, 30.1 ± 4.4% at Month 6, 31.5 ± 3.7% at Month 12 of ERW treatment and 33.8 ± 4.6% at Month 6 post-ERW treatment. The administered dosages of erythropoietin per kilogramme body weight were 278 ± 167 U/kg/month at Month 0, 335 ± 165 U/kg/month at Month 1, 330 ± 202 U/kg/month at Month 3, 298 ± 167 U/kg/month at Month 6, 315 ± 197 U/kg/month at Month 12 of ERW treatment and 282 ± 177 U/kg/month at Month 6 post-ERW treatment.

It has been suggested that HD enhances T-cell activation-induced cell death. We evaluated the percentage of apoptosis (annexin $V^+$–$PI^+$) and necrosis (annexin $V^-$–$PI^+$) in the T lymphocytes from the ESRD patients undergoing HD. Our results showed that a significant increase in apoptosis (annexin $V^+$–$PI^+$) and necrosis (annexin $V^-$–$PI^+$) in the pre-dialysis T cells was found in the patients when compared to the healthy subjects (Figure 1). ERW treatment significantly reduced the percentage of annexin $V^+$–$PI^+$ and annexin $V^-$–$PI^+$ when compared to the pre-ERW levels. The decreased percentage of T-cell apoptosis and necrosis tended to recover at Month 6 post-ERW treatment.

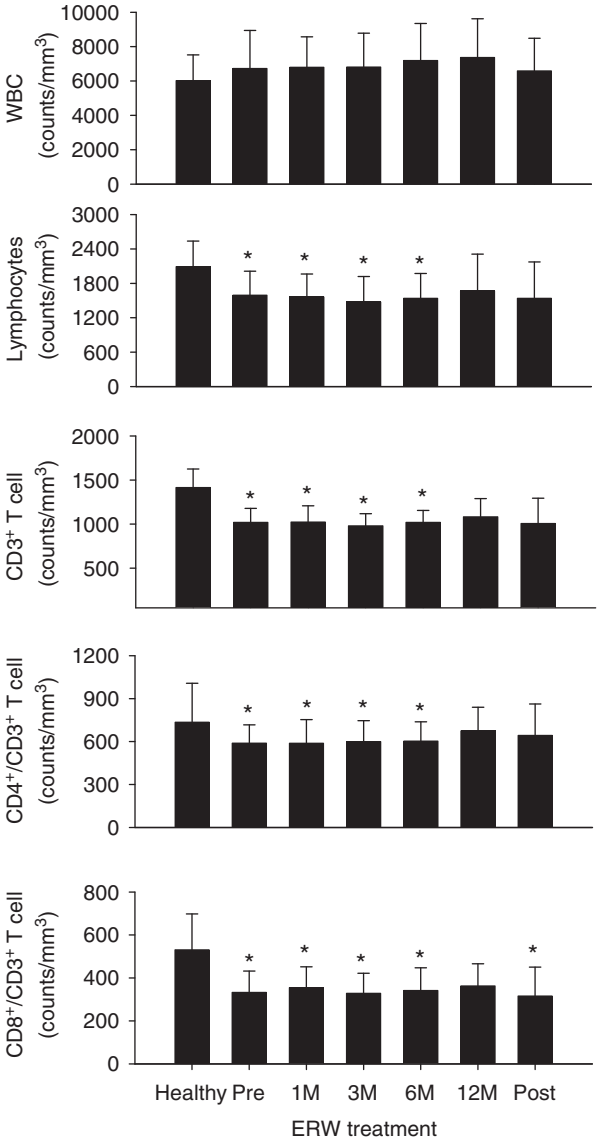


**Fig. 2.** ERW on leukocytes (WBC) count and T cell subsets. Absolute counts of WBC, lymphocytes, $CD3^+$, $CD4^+/CD3^+$ and $CD8^+/CD3^+$ T cells in the patients ($n$ = 42) prior to (Pre), during the first month (1M), third month (3M), sixth month (6M) and 12th month (12M) of ERW treatment and 6 months post-ERW treatment (Post). ERW treatment mildly but significantly increased the numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T cells at 12 months of treatment. At 6 months post-ERW treatment, lymphocytes, $CD3^+$, $CD4^+/CD3^+$ and $CD8^+/CD3^+$ T cells in the patients tended to decrease. Mean ± SD. *P < 0.05 when compared to healthy controls ($n$ = 12).

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When compared to healthy controls, the ESRD patients with chronic HD had decreased $CD3^+$, $CD4^+$ and $CD8^+$ T-cell counts (Figure 2). ERW treatment mildly increased the counts of $CD3^+$ and $CD8^+$ T cells in the patients. After ERW treatment had been stopped for 6 months, the counts of $CD4^+$ and $CD8^+$ T cells in the patients tended to decrease.

In comparison to the healthy controls, the percentage of surface marker expressions of $CD25/CD3^+$, $CD69/CD3^+$ and $CD94/CD3^+$ cells were all significantly reduced in the ESRD patients with chronic HD (Figure 3). One-year ERW treatment significantly ($P < 0.05$) increased the percentage of $CD25/CD3^+$, $CD69/CD3^+$ and $CD94/CD3^+$ expressions in the patients. However, further increases in $CD25/CD3^+$, $CD69/CD3^+$ and $CD94/CD3^+$ phenotypes were found at 6 months post-ERW treatment.

The intracellular levels of the Th1/Tc1 cytokines, IL-2 and IFN-γ, in $CD4^+$ and $CD8^+$ cells were significantly ($P < 0.05$) decreased in the ESRD patients with chronic HD when compared to the healthy subjects (Figures 4 and 5). In contrast, the intracellular levels of Th2/Tc2 cytokines, IL-4, IL-6 and IL-10, in $CD4^+$ and $CD8^+$ cells were all significantly increased in the patients.

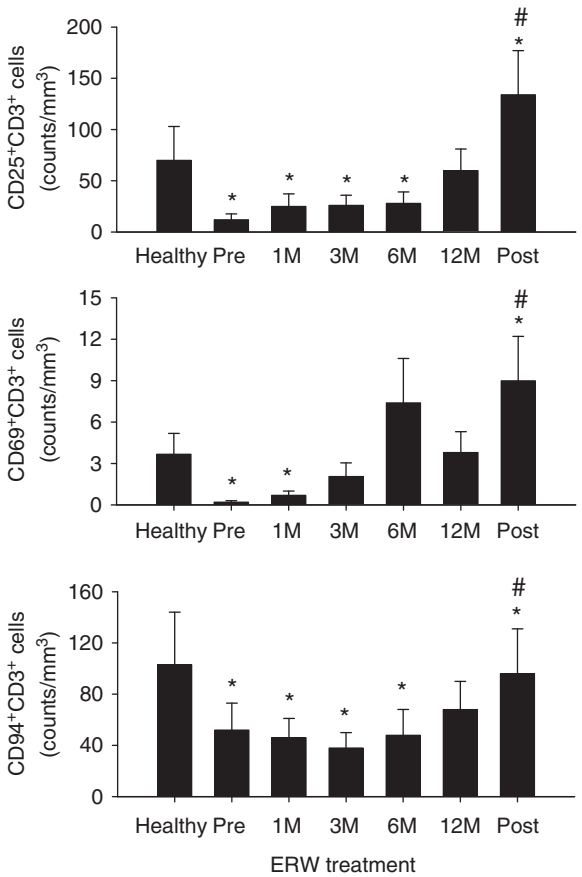


**Fig. 3.** ERW on CD25-related surface markers. Effect of 12 months of ERW treatment on CD25, CD69 and CD94 surface markers in $CD3^+$ cells from the HD patients ($n = 42$). ERW treatment increased $CD25^+CD3^+$, $CD69^+CD3^+$ and $CD94^+CD3^+$ cell counts during the first month (1M), third month (3M), sixth month (6M) and 12th month (12M). At 6 months post-ERW treatment (Post), CD25, CD69 and CD94 surface markers were further upregulated in $CD3^+$ cells. Mean ± SD. $*P < 0.05$ when compared to healthy controls ($n = 12$); #$P < 0.05$ Post *vs* 12M.

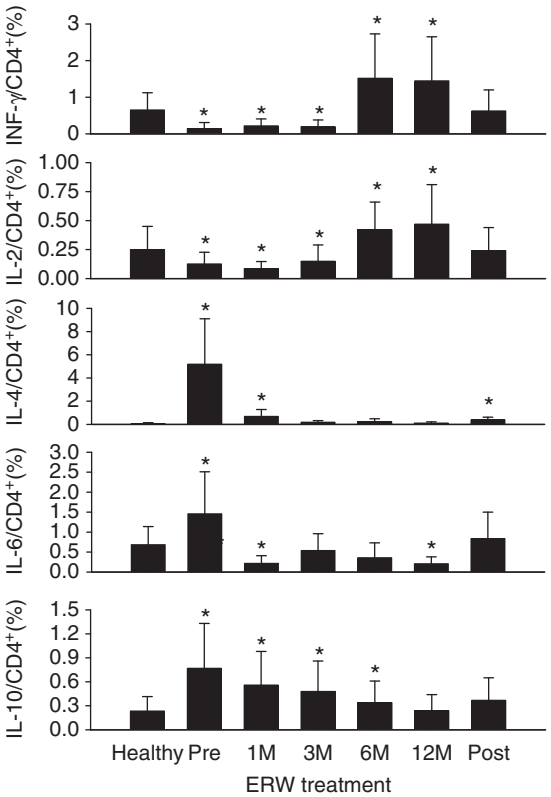


**Fig. 4.** ERW on Th1/Th2 cytokine profiles. Effect of 12-month ERW treatment on intracellular Th1/Th2 cytokine profiles in $CD4^+$ T cells from the patients ($n = 42$) prior to (Pre), at the first month (1M), third month (3M), sixth month (6M) and 12th month (12M) of ERW treatment and 6 months post-ERW treatment (Post). ERW treatment significantly increased the percentage (%) of INF-γ and IL-2 (Th1 cytokines) expressions in $CD4^+$ T cells during 6–12 months of ERW treatment. In contrast, ERW treatment significantly depressed IL-4, IL-6 and IL-10 (Th2 cytokines) expressions during 1–12 months of ERW treatment. At 6 months post-ERW treatment, Th1 cytokines tended to decrease and Th2 cytokines tended to increase in the patients. Mean ± SD. $*P < 0.05$ when compared to healthy controls ($n = 12$).

The long-term effect of ERW treatment on Th1 and Th2 cytokine profiles in $CD4^+$ T cells from the ESRD patients with chronic HD is demonstrated in Figure 4. One-year ERW treatment significantly increased INF-γ and IL-2 expressions. In contrast, ERW treatment significantly depressed IL-4, IL-6 and IL-10 expressions. At 6 months post-ERW treatment, INF-γ and IL-2 expressions tended to decrease and IL-4, IL-6 and IL-10 expressions tended to increase in the patients.

The effect of ERW treatment on Tc1 and Tc2 cytokine profiles in $CD8^+$ T cells from the ESRD patients with chronic HD is displayed in Figure 5. Long-term ERW treatment significantly increased INF-γ and IL-2 expressions. ERW treatment significantly depressed IL-4, IL-6 and IL-10 expressions. At 6 months post-ERW treatment, INF-γ and IL-2 expressions tended to decrease and IL-4, IL-6 and IL-10 expressions tended to increase in the patients. We further compared the Th1/Th2 and Tc1/Tc2 ratios between the ESRD patients and healthy subjects. Long-term ERW treatment mildly but significantly

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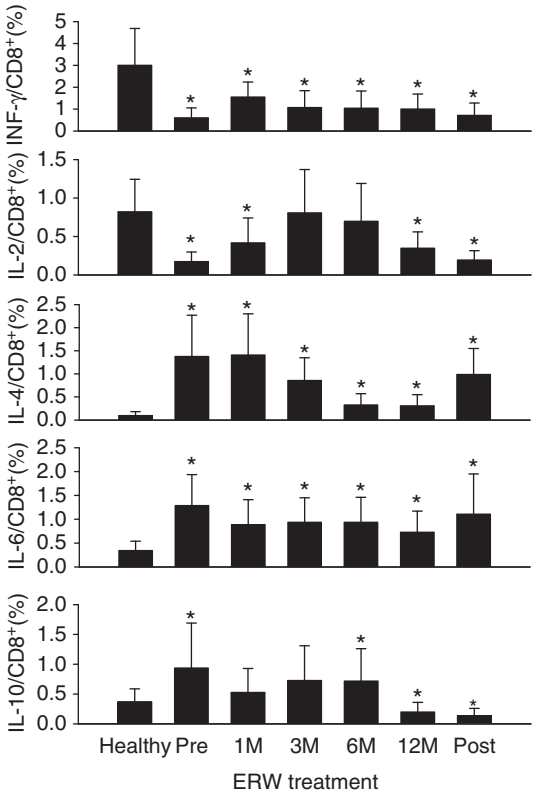


**Fig. 5.** ERW on Tc1/Tc2 cytokine profiles. Effect of 12-month ERW treatment on intracellular Tc1/Tc2 cytokine profiles in $CD8^+$ T cells from the patients ($n$ = 42) prior to (Pre), at the first month (1M), third month (3M), sixth month (6M) and 12th month (12M) of ERW treatment and 6 months post-ERW treatment (Post). ERW treatment significantly increased the percentage (%) of INF-γ and IL-2 (Tc1 cytokines) expressions in $CD8^+$ T cells during 1–12 months of ERW treatment. In contrast, ERW treatment significantly depressed IL-4, IL-6 and IL-10 (Tc2 cytokines) expressions during 12 months of ERW treatment. At 6 months post-ERW treatment, Tc1 cytokines tended to decrease and Tc2 cytokines tended to increase with the exception of IL-10 in the patients. Mean ± SD. *P < 0.05 when compared to healthy controls ($n$ =12).

increased the Th1/Th2 and Tc1/Tc2 ratios toward a normal status. At 6 months post-ERW treatment, the Th1/Th2 and Tc1/Tc2 ratios tended to decrease.

## Discussion

Our study indicates that 12 months of ERW treatment decreased T-cell apoptosis, increased the absolute numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes and CD25/CD69/CD94/$CD3^+$ surface marker expressions and modulated Th1/Th2 and Tc1/Tc2 intracellular cytokine ratios toward a normal status.

In 1985, Hayashi proposed the 'Water Regulating Theory' for the clinical improvement of diseases by intake of reduced water [27]. The ideal ROS scavenger could be active atomic hydrogen, which can be produced in ERW during water electrolysis. ERW exhibits a high pH, low dissolved oxygen, rich atomic hydrogen and an extremely negative redox potential value [25]. ERW with superoxide dismutase- and catalase-like activities can scavenge ROS [25]. Happe *et al*. implicated that hydrogenases, which are among the oldest enzymes (3.8 billion years old), can reversibly split molecular hydrogen to produce active atomic hydrogen contributing to the redox regulation of cellular function [26]. The ROS scavenging capability of ERW is stable at 4 °C for over a month and not lost even after neutralization, repeated freezing and melting, deflation with sonication, vigorous mixing, boiling, repeated filtration or closed autoclaving, but is lost by opened autoclaving or by closed autoclaving in the presence of tungsten trioxide which efficiently adsorbs active atomic hydrogen [25]. Rich active hydrogen in ERW is an ideal ROS scavenger because it does not produce oxidized molecules after reduction like other organic antioxidants (vitamin C, vitamin E and polyphenols) [1,3,8,29]. ERW, vitamin C and catechins completely scavenge $O_2^{-\bullet}$ produced by the hypoxanthine–xanthine oxidase system in a sodium phosphate buffer [25]. Furthermore, ERW, catalase and vitamin C can directly scavenge $H_2O_2$ [1,3,25]. We found that ERW and catechins can scavenge both $H_2O_2$ and HOCl, but vitamin C only removes $H_2O_2$ [1,29]. The reduction in plasma HOCl activity may preserve HD-decreased serum paraoxonase 1 activity [29], an HDL-associated oxidant-sensitive enzyme which can inhibit the atherogenic oxidation of LDL. The scavenging ROS mechanism by ERW may be due to the direct adsorption of ROS by active atomic hydrogen because no antioxidant production was detected by a total antioxidant status assay in our previous studies [1,3]. Our serial studies [1,3] have indicated that ERW can be used in HD patients due to its stable characteristics, lower cost and strong antioxidant activity to reduce HD-evoked oxidative stress, proinflammatory cytokine levels and atherosclerotic risk factors and preserve the activity of erythrocyte reductases and increase haematocrit during 6 months of treatment. During our previous study with 6 months of ERW dialysate in HD patients, levels of biochemical parameters were stable and no long-term toxicity/adverse effects on the liver, kidney and other organs were noted. ERW treatment can also replace intravenous vitamin C infusions to prevent vitamin C overdoses and secondary oxalosis manifested by an increase in plasma oxalate levels and the deposition of calcium oxalate in various tissues [1,8,30].

The oxidative stress associated with uraemia is exacerbated by HD because neutrophils and monocytes, activated by contact with the dialysis membranes, release large amounts of ROS [1,3,7,8,29]. 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 [1,3,8,13,29]. In contrast to healthy status, ESRD induces a clinical state of immunodeficiency with a higher incidence of infections and higher mortality due to infectious complications. Furthermore, ESRD patients, especially when treated with HD, have increased oxidative stress in T lymphocytes, which may lead to abnormal cytokine synthesis and immune response. The results of this study implicate that ERW treatment reduces T-cell priming as the cause of intracellular ROS and low-grade inflammation associated with ESRD. The augmented ROS release together with the increased intracellular Th2/Tc2 cytokines and DNA-bound NF-κB/p65 and AP-1/c-Fos activities are known contributors to systemic oxi-

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dative stress. We previously showed that systemic inflammatory markers such as CRP, IL-6 and TNF-α positively correlate with plasma $H_2O_2$ activity [1,3,8,29]. Our previous results further showed that 26 proinflammatory cytokines are downregulated after 6 months of ERW treatment [1]. These findings provide a new insight into the molecular events that resolve inflammation and immunodeficiency in chronic HD patients by ERW treatment.

$CD8^+$ T cytotoxic lymphocytes can be the first line of immunodefence by production of several cytokines such as IFN-γ after infection and before T-cell proliferation, contributing to the host-acquired immunity [31]. CD25 (IL-2 receptor) is expressed in functional T lymphocytes, and then upregulation of CD25 can trigger $CD8^+$ T cytotoxic lymphocyte clonal expansion and immune response [32]. Downregulation of CD25 has been found in humans with colon cancer, breast cancer and renal carcinoma [32]. CD69 is expressed in the activated T and B lymphocytes and natural killer cells. CD69 can drive the clonal expansion, cytokine release and cytotoxicity in T lymphocytes and natural killer cells. CD25 can be negatively regulated by CD94/NKG2A receptors to prevent overt activation and killing of the host cells [33]. Upregulation of CD94/NKG2 can prevent T-lymphocyte activation-induced apoptotic cell death [34]. In clinical trials, high expressions of CD94 prolonged the survival rates in nasal-type extranodal NK/T cell lymphoma patients [35] or prevented leukocyte activation-induced apoptosis [34]. Upregulation of CD94/NKG2A can increase the sensitivity threshold to antigen stimulation and delicately control cytotoxic T-lymphocyte response, therefore, preventing antigen activation-induced cell death and clonal exhaustion and promoting the generation of a pool of memory cells [36,37]. In our study, ERW treatment significantly increased CD25, CD69 and CD94 expressions in the T lymphocytes. After ERW treatment had been stopped for 6 months, a higher activation of CD25, CD69 and CD94 were found. These data suggest that upregulation of CD94 in T lymphocytes can delicately control the upregulation of CD25 (IL-2 receptor) and CD69 in the activated T lymphocytes after ERW treatment. The immunomodulation by ERW treatment might improve the activation-induced cell death in the T lymphocytes of ESRD patients undergoing chronic HD. These data also show that, during ERW treatment, an upregulation in $CD25/CD3^+$ and $CD69/CD3^+$ may implicate a functional response in T cells. The significant elevation of $CD25/CD3^+$, $CD69/CD3^+$ and $CD94/CD3^+$ at 6 months post-ERW treatment may impact a negative regulation of enhanced $CD25/CD3^+$ and $CD69/CD3^+$ by the upregulation of $CD94/CD3^+$. ERW may prevent T cells exaggerated priming after chronic HD.

T-cell activity is significantly retarded in ESRD patients compared with normal subjects. The proportions of T cytotoxic cells are significantly higher in HD patients, whereas the proportions of T helper/inducer and B cells are significantly lower in HD patients than in controls [38]. In our study, 12 months of ERW treatment mildly increased the absolute numbers and the proportions of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes in the ESRD patients with chronic HD. These data suggest that long-term ERW treatment may provide an improvement and prevention of HD-enhanced T-cell death in ESRD patients.

To detect polarized Th1/Th2 or Tc1/Tc2 responses, intracellular staining by flow cytometry methods is relatively simple and highly sensitive. Th1/Th2 and Tc1/Tc2 cytokines are implicated in regulating the immune responses and may be involved in impaired status. $CD4^+$ T cells produce IFN-γ, IL-2, IL-4 and IL-10 in response to infected macrophages, whereas $CD8^+$ T cells produce predominantly IFN-γ and IL-2. Since IL-4 and IL-10 can suppress IFN-γ-mediated protective mechanisms against parasites, the production of these cytokines by $CD4^+$ T cells in response to infected cells can negatively affect their protective activity *in vivo* [39]. The production of plasma IL-2, which is involved in cell-mediated immune responses, and the production of plasma IL-4 and IL-10, which affects humoral immunity, are significantly lower in HD patients than in controls [38]. Both $CD4^+$ T (Th) and $CD8^+$ T (Tc) lymphocytes can be functionally divided into type 1 and type 2 subsets based on the secretion of either IFN-γ (T1) or IL-4 (T2), while the synthesis of cytokines such as IL-2, IL-6 and IL-10 is not stringently restricted to a single subset [40]. Previous results have demonstrated that both cellular immunity induced by Th1 and humoral immunity induced by Th2 decrease in HD patients [38]. The T-cell activity with the production of IL-2, IL-4 and IL-10 was significantly reduced in HD patients compared to controls [38]. In the present study, we found that a lower percentage in the Th1 and Th2 cytokines of $CD4^+$ cells and Tc1 and Tc2 cytokine expression of $CD8^+$ T cells was found in the ESRD patients with chronic HD. One possibility for the lower value of intracellular cytokine expression in the $CD4^+$ and $CD8^+$ T cells was due to a short period of 4 h of PMA and ionomycin stimulation. On the other hand, although stimulation with PMA and ionomycin usually gives higher percentage of T cells producing cytokines, especially for Th1 or Tc1 cytokines when compared to other diseases [41], we suggest that renal insufficiency and chronic HD contribute to the lower expression of intracellular cytokines in the T cells. However, these required further studies. ERW treatment significantly increased INF-γ and IL-2 (Th1 and Tc1 cytokines) levels during 12 months of ERW treatment. In contrast, ERW treatment significantly depressed IL-4, IL-10 and IL-6 during the 12 months of ERW treatment. Six months after ERW treatment had been stopped, INF-γ and IL-2 levels and IL-4 and IL-10 levels decreased, but an increase in IL-6 level was noted in these ESRD patients with chronic HD. The decrease in Th1/Th2 and Tc1/Tc2 ratios in comparison to healthy controls could be partly reversed by long-term ERW treatment. According to the medical records, the yearly rates of hospitalization due to infections were one out of 42 (2.38%), one out of 42 (2.38%) and zero out of 42 (0%) prior to, at the course of and post-follow-up stage, respectively. These findings suggest that long-term ERW treatment may diminish the infection rate of ESRD patients with chronic HD.

## Conclusion

In conclusion, our findings demonstrate that long-term ERW treatment attenuates chronic HD-evoked immunodysfunction in T lymphocytes, as indicated by a decrease

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in T-lymphocyte apoptosis, an increase in CD25-related surface marker expression in the T cells and a shift in cytokine profiles from Th2/Tc2 to Th1/Tc1 status in the ESRD patients undergoing chronic HD.

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