CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and Reagents

The details of chemicals and reagents are shown in Appendix.

2.2 Patients

One hundred and ten end-stage renal disease (ESRD) out-patients at Kawila Hospital (n = 100), Nakhornping Hospital (n = 9) and Chang-Puek Hospital (n = 1) in Chiang Mai were recruited for this study. The ages of patients were between 25-85 years and they were regularly treated with 4-hours hemodialysis. They were divided into two groups. Group ESRD I had been treated with subcutaneous injection of recombinant human erythropoietin (rHuEPO) or Eprex® hormone and oral ferrous sulfate tablets, Group ESRD II had been treated with blood transfusion and oral ferrous sulfate tablets. None of them has been administered with any iron chelator. The control group consisted of 15 healthy volunteers with ages between 21-24 years and showed a normal renal function.

2.3 Blood samples

Whole blood from the ESRD patients was taken prior to the hemodialysis session, collected into a lithium heparin-coated vacutainer tube and mixed gently. Heparinised blood was divided into two aliquots, which one is still whole blood and another one is for plasma preparation. Plasma was obtained by centrifugation at 4,000 rpm (1180 x g) for 10 min at 4 ^{o}C, separated and kept frozen at -20 ^{o}C until analysis.

2.4 Determination of renal function

Renal function was determined by blood urea nitrogen (BUN) level based on the Fearon method. In principle, urea condenses directly with diacetyl under high temperature to form a pink-colored diazine. Because diacetyl is unstable, it is usually generated in the reaction system from diacetyl monoxime and acid (Rock, *et al* 1987). The reaction of diacetyl and urea gives diazine as follows:

$$\text{Diacetyl monoxide} \xrightarrow{\textit{Acid}} \text{Diacetyl}$$

$$\text{Diacetyl} + \text{Urea} \xrightarrow{\textit{Heat}} \text{Diazine}$$

In the assay, 3 ml. of oxime solution was mixed with 3 ml. of acid solution in test tube. Subsequently, 0.02 ml. of plasma sample or standard urea nitrogen solution was added into test tube, the contents of tube was thoroughly mixed and heated in water bath at 100 ^{O}C for 10 min. After cooling, the optical density (OD) was measured at 520 nm., using oxime solution and acid solution as a reagent blank to set zero absorbance.

2.5 Determination of red blood cell concentration

2.5.1 Hemoglobin determination

The measurement of blood hemoglobin (Hb) is based on cyamethemoglobin method. In this method, hemoglobin in red blood cells is firstly oxidised by ferricyanide in Drabkin's reagent to form methemoglobin. The intermediate subsequently complexes with cyanide in the same reagent to produce a very stable colored end-product, cyanmethemoglobin (Fairbanks and Klee 1987).

$$\text{Hb}\bullet\text{Fe(II)} + \text{Fe(III)(CN)}_6^{3-} \longrightarrow \text{Hb}\bullet\text{Fe(III)} + \text{Fe(II)(CN)}_6^{4-}$$

$$\text{Hb}\bullet\text{Fe(III)} + \text{CN}^- \longrightarrow \text{Hb}\bullet\text{Fe(III)(CN)}$$

In the assay, 0.02 ml. of whole blood sample was pipetted into test tube, 5 ml. of Drabkin's reagent was then dispensed into the same test tube. Contents of tube was mixed thoroughly and let stand at room temperature for at least ten minutes. The OD was measured at 540 nm., using the Drabkin's solution as a reagent blank to set zero absorbance. Accuglobin® at various concentrations was used as a standard hemoglobin solution. Standard curve was made by plotting different hemoglobin concentrations on the abscissa (x-axis) against the absorbance unit (AU) on the ordinate (y-axis).

2.5.2 Hematocrit determination

The test is based on the principle of separating the cellular elements of the blood from plasma, which the separation process is accelerated up by high-speed centrifugation. After centrifugation of blood in a tube, the red blood cells will be at the bottom of the tube whereas the white blood cells and platelets will form a layer on top of the red cells, and the plasma will be at the top. Then, hematocrit (Hct) value is determined by comparing the volume of red blood cells to the total volume of the whole blood sample. Generally, it is expressed as the percentage of obtained Hct value.

In the assay, blood sample was drawn into a vacutainer tube containing lithium heparin as an anti-coagulant, gently mixed, then sucked into a capillary tube by capillary action. One end of capillary tube was firmly closed with sealing clay. The sealed tube was placed in a hematocrit centrifuge and the lid to the centrifuge was secured. The tubes were centrifuged at 10,000 rpm. for 5 minutes. Using a hematocrit reader, hematocrit value was determined by placing the tubes on a hematocrit reader.

2.6 Determination of iron overload status

2.6.1 Plasma iron determination

The principle of this method is based on iron released from transferrin by a decrease in pH of plasma. Proteins and apotransferrin are removed with a precipitation with trichloroacetic acid (TCA) and centrifugation. The Fe (III) of the supernatant is reduced to Fe(II) with thioglycolic acid and is complexed with a chromogen. The absorbance of the iron-chromogen complex is proportional to the iron concentration in the plasma. Buffer solution is added to adjust the pH to 4.5 to ensure maximum absorption of the iron-chromogen complex (ICSH 1978). If bathophenanthroline is used as the chromogen , the reactions involved are:

$$\text{Transferrin} \bullet \text{Fe(III)} + \text{HCl/Trichloroacetic acid} \longrightarrow \text{Fe(III)} + \text{Transferrin} (\downarrow)$$

$$\text{Fe(III)} + \text{Thioglycolic acid} \longrightarrow \text{Fe(II)}$$

$$\text{Fe(II)} + \text{Bathophenanthroline (colorless)} \longrightarrow \text{Bathophenanthroline} \bullet \text{Fe(II) (colored)}$$

In the procedure, 0.2 ml of plasma was mixed with 0.2 ml of 10% TCA solution as a protein-precipitating solution in centrifuge tube, allow to stand at room temperature for 5 minutes and subsequently centrifuged at 6,000 rpm, 4 ^{o}C for 10 minutes to achieve the clear supernatant. 0.2 ml of standard iron

solution at different concentrations was mixed with 0.2 ml of 10% TCA solution in a centrifuge tube and allowed to stand at room temperature for 5 minutes. Similarly, 0.1 ml of deionised water was added to a labeled blank tube and 0.1 ml of 10% TCA solution was mixed and allowed to stand at room temperature for 5 minutes. Each solution was separately transferred in a duplicate to the wells labeled as the reagent blank, unknown sample and standard in a Dynatech® 96-well microtitre plate. Finally, 0.1 ml of the chromogen solution was added to each well and allowed to stand at room temperature for 5 minutes for colour development. The absorbance of unknown and standard solutions was read within 30 minutes against reagent blank at 540 nm using a microtitre plate reader.

2.6.2 Total iron-binding capacity determination (TIBC)

Total iron-binding capacity is a measurement of the maximum concentration of that plasma protein, particularly transferrin, can bind iron. Serum TIBC varies in disorders of iron metabolism. The principle of this method is based on excess ammonium ferric citrate that is incubated with serum to saturate all iron-binding sites of transferrin. The unbound Fe(III) is removed by addition of $MgCO_3$ powder, which it adsorbs excess Fe(III)] and a buffer (ICSH 1978b). The mixture is centrifuged and the iron content of the supernatant is assayed according to plasma iron determination as previously described in **Section 2.6.1**.

In the assay, 10 µl of ammonium ferric citrate solution was incubated with 0.5 ml of plasma at room temperature for 30 minutes 0.4-0.5 g of light magnesium carbonate was then added, mixed on a rotary mixer for at least 15 minutes and centrifuged at 6,000 rpm, 4 ^{o}C for 10 minutes 0.2 ml of supernatant was transferred to another tube, proceeded with 0.2 ml of 10% TCA and allowed to stand at room temperature for 5 minutes for a complete protein precipitation. The mixture was centrifuged at 6,000 rpm, 4 ^{o}C for 10 minutes, 0.1 ml of the supernatant was subsequently transferred to the well of 96-well microtitre plate. For a reagent blank tube, 0.1 ml of deionised water was incubated with 0.1 ml of 10% TCA and allowed to stand at room temperature for 5 minutes 0.1 ml of chromogen solution was later added to each well and allowed to stand at room temperature for 5 minutes. Finally, the absorbance was read against a reagent blank at 540 nm using a microtitre plate reader.

2.6.3 Percentage of transferrin saturation determination

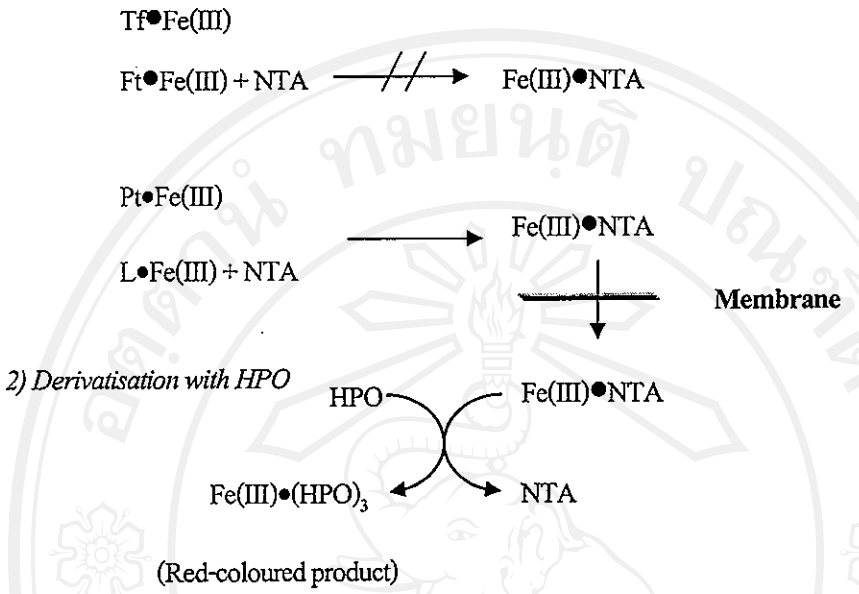
Transferrin saturation is calculated by using a formula as following

Transferrin saturation (%) = (plasma iron/TIBC) x100

2.7 Determination of plasma non-transferrin bound iron (NTBI)

In principle, NTBI but neither transferrin- nor ferritin-bound iron in the plasma is first specifically chelated by a weak tridentate iron chelator, nitrilotriacetic acid (NTA), to form a Fe^{3+}-$(NTA)_2$ complex, which it will be subsequently removed from plasma proteins by centrifugation through a special membrane filter. Using the reverse-phase HPLC technique, this complex will be immediately on-column derivatised with a strong bidentate 3-hydroxypyridin-4-one (HPO) iron chelator, 1,2-dimethyl-3-hydroxypyridin-4-one (CP20 or L1) or 1-propyl-2-methyl-3-hydroxypyridin-4-one (CP22), to produce a red colored product Fe^{3+}-$(HPO)_3$ that is Fe^{3+}-$(CP20)_3$ or Fe^{3+}-$(CP22)_3$ respectively. Based on a hydrophobic property of octadecylsilane (ODS of C18) materials, the red colored Fe^{3+}-$(CP20)_3$ and Fe^{3+}-$(CP22)_3$ complexes can be separated from other compounds by an isocratic elution with 5% acetonitrile at approximately 1.5 minute retention time or 3.4 minute retention time respectively. Finally, this product is detected at the wavelength of 450 nm (Singh et al., 1990; Al-Rafaie et al., 1992). Using this technique, iron present in buffer system is mopped up with an excess CP20 or CP22 in the mobile-phase solvent. Iron contaminated in the membrane filters and plastic containers needs to be cleaned up with 10 mM NTA solution before use (Singh, *et al* 1990). A brief assay chart is shown below (Figure 2.1).

1) Chelation with NTA



(Ft = ferritin, Tf = transferrin, NTA = nitrilotriacetic acid, Pt = protein, L = low-molecular-weight ligand)

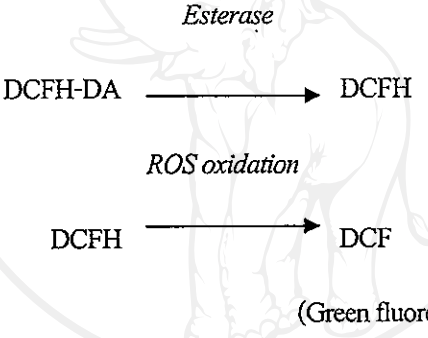
Figure 2.1 Diagram of plasma NTBI quantification using the NTA chelation/HPLC-based assay.

In the assay, 50 µl of 800 mM NTA, pH 7.0 solution was added to 0.45 ml of plasma, incubated at room temperature for 30 minutes to form a Fe^{3+}-$(NTA)_2$ complex. This complex was separated from plasma proteins and forced into the filtrate by centrifugation on a pre-washed polysulfone membrane filters (NanoSep 30*k*D Omega, P/N OD030C33. Pall Corporation Michigan, U.S.A.) at 12,000 rpm, 15 ^{o}C for 45 minutes. The filtrate was injected into a Rheodyne® 50-µl loop of HPLC system equilibrating at a flow rate of 0.6 ml/min in a mobile-phase solvent (3 mM 1,2-dimethyl-3-hydroxypyridin-4-one or CP20 in 5% acetonitrile buffered with 5 mM MOPS, pH 7.0), fractionated on the HPLC glass column (ChromSpher C18, 100x3mm, 5 µm, Cat. No. CP28267, Varian U.S.A.) and on-column derivatised with CP20 in the mobile-phase solvent to form a Fe^{3+}-$(CP20)_3$ complex immediately. Absorbance or peak height (PH) of the eluted Fe^{3+}-$(CP20)_3$ product was measured at 450 nm. Plasma NTBI concentration was calculated from the calibration curve made from different standard iron concentrations (0, 2, 4, 8 and 16 µM Fe^{3+} in 80 mM NTA). Normal and thalassemic plasma were included in the assay as the biological control.

2.8 Determination of oxidative stress status

2.8.1 Determination of oxidative stress in RBC by flow cytometry

Flow cytometry has been used to measure oxidative stress in various types of cells. With many advantages, this method is fast, accurate, very sensitive and applicable for several kinds of cells. Various compounds have been tested for measuring ROS generation demonstrated as the fluorescence of their oxidized derivatives. Among them, the suitable fluorochromes are dihydrorhodamine123 and 2',7'-dichlorofluorescin diacetate (DCFH-DA). Upon crossing the cell membrane, the latter compound DCFH-DA is trapped inside the viable cells and hydrolysed by intracellular esterase to produce a DCFH compound. Oxidation of DCFH by ROS produces the highly fluorescent 2,7'-dichlorofluorescein (DCF). By measuring the cellular fluorescence intensity, the rate and extent of ROS production can be quantified accurately and rapidly (Amer, *et al* 2003).



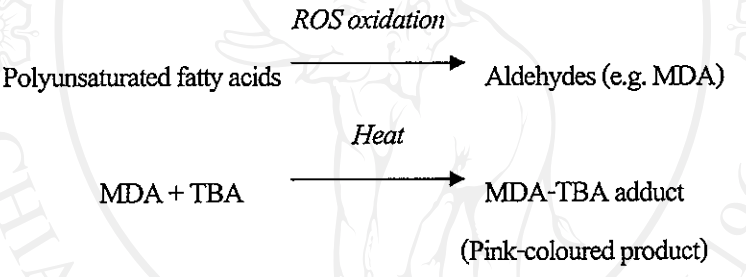
In the assay, heparinzed peripheral blood (2 µl) obtained from both normal volunteers and end-stage renal disease (ESRD) patients was diluted with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) solution to achieve a suspension of $1x10^6$ RBCs/ml. DCFH-DA (Sigma, St Louis, MO, USA) solution, which was previously dissolved in methanol, was added at a final concentration of 0.4 mM to the cells and incubated at 37 ^{o}C for 15 minutes in a humidified atmosphere of 5% CO_2 in air. Cells were centrifuge at 4,000 rpm, 10 ^{o}C for 10 minutes and the supernatant was aspirated. RBC pellet was twice washed in 2 ml of PBS at 4,000 rpm, 10 ^{o}C for 10 minutes, and reconstituted in 1 ml of PBS. The sample was then divided into 0.5-ml fraction to further incubate in the dark at room temperature for 20 minutes without and with 3% H_2O_2 stimulation (35 µl) that was freshly prepared before each experiment by diluting a 30% stock solution with PBS. Finally, the labeled RBCs were analyzed with a Fluorescence Activated Cell Sorter (FACSort, Becton-Dickinson, Immunofluorometry systems, Mountain View, CA, USA) by being passed at rate of about 800 per second, using DAKO sheath fluid. A 488-nm argon laser beam was used for excitation. A two-parameter dot-plot of the side light scatter (SSC) and forward light scatter (FSC) of the population was

first analyzed and FL-1 was included. The geometric mean of measured fluorescence intensity was derived by CellQuest® software that had been ready-installed in a PowerMac computer. Percentage of control (% of control) was determined by using the formula as below.

$$\% \text{ of control} = \frac{\text{FI of curcumin or Fe-EDTA treated- sample}}{\text{FI of PBS treated- sample}} \times 100$$

2.8.2 Determination of thiobarbituric acid-reactive substances (TBARS) in plasma

Malondialdehyde (MDA) is the most abundant aldehyde arising from lipid peroxidation. It is determined by measurement of colored product formed upon reaction with thiobarbituric acid (TBA). TBARS determination is one of the most common and easy assays used in lipid peroxidation studies. The tested sample is treated with TBA in acidic pH and a pink chromogen is measured (Gutteridge 1981).



Determination of TBARS was performed as following. 0.25 ml of plasma or standard MDA was first pipetted into test tube, and then 25 µl of 0.2% BHT was added. Tube contents were mixed thoroughly, and the sample mixture was divided into 137-µl aliquots. Afterwards, 0.75 ml of 0.44 M H_3PO_4 was added and left at room temperature for 10 minutes. Finally, 0.25 ml of 0.6% TBA was added and the reaction was initiated by heating at 90 ^{o}C for 30 minutes. For reagent blank, deionised water was added instead of 0.6% TBA. After cooling, the absorbance of unknown and standard MDA solutions was measured against reagent blank at 540 nm using a microtitre plate reader.

2.9 Effect of hemodialysis on NTBI level

2.9.1 Effect of *in vivo* hemodialysis by hemodialyzer

Logically, NTBI is present in the plasma of patients with β-thalassemia major and HbE when transferrin saturation is increased due to the excess iron from multiple blood transfusions and disorder of iron absorption. This situation probably occurs in some ESRD patients who are deficient in erythropoietin

(EPO) synthesis and consequently defective in the erythropoiesis. To maintain the level of blood hemoglobin above 12 g/dl, some patients are regularly administered oral iron tablets or intravenous iron injection together with subcutaneous EPO injection while orther patients have received intermittent blood transfusions. Since they have not been received any iron chelators and evaluated their iron status, NTBI may appear in their blood circulation resulting in the ROS formation and tissue damage. Hemodialysis is an effective routine method to get rid of waste products like urea, creatinine and phosphate persisting in their blood; nevertheless, no body knows whether plasma NTBI could be removed by this way or not.

Herein, it is interesting to examine the *in vivo* effect of hemodiaysis by hemodialyzer on NTBI level in ESRD patients. In the assay, 5 ml of whole blood from five ESRD patients with iron overload status was collected into lithium heparin-coated vacutainer tubes before and after 4-hour hemodialysis. NTBI level was measured using the HPLC method as described in **Section 2.7.**

2.9.2 Effect of *in vitro* hemodialysis

To examine the effect of hemodialysis *in vitro* on NTBI level, 10 ml of the whole blood from the same ESRD patients in **Section2.9.1** was first collected into lithium heparin-coated tube and divided into two tubes (1.2 ml each). PBS was added into Tube 1 (control) and deferiprone (a final concentration of 100 μM in MOPS buffer, pH7.0) was added into Tube 2. Each solution was transferred to dialysis-membrane bags (Sigma-Aldrich Chemical Co., serial number D-9652, pore size 12 *k*D, average flat with 33 mm, average diameter 21 mm, capacity approximately 110 ml/ft) separately and performed *in vitro* diaysis in 250 ml of working dialysate solution at room temperature for 4 hours. NTBI concentration was measured using the HPLC method as previously described in **Section 2.7.**

2.9.3 Effect of curcumin and deferiprone on *in vitro* hemodialysis

To examine the effect of hemodialysis *in vitro* on NTBI level, 10 ml of the whole blood from the same ESRD patients in **Section 2.9.1** was first collected into lithium heparin-coated tubes and divided into four tube sets (1.2 ml each). PBS was added into Tube 1 (control) and deferiprone (a final concentration of 100 μM in MOPS buffer at pH 7.0) together either with curcumin at a final concentration 20, 100, 800 μM in MOPS buffer at pH7.0 was added into tube 2, 3 and 4. Each solution was transferred to dialysis-membrane bags separately and performed *in vitro* diaysis in 250 ml of working dialysate solution at room temperature

for 4 hours. NTBI concentration was measured using the HPLC method as previously described in **Section 2.7.**

2.10 Effect of curcumin on iron-binding activity *in vitro*

2.10.1 Spectral analysis

Curcumin solution (a final concentration of 800 μM in MOPS buffer at pH7.0) was incubated with ferric nitrate or ferric citrate (20, 40, 80 and 100 μM at final concentrations) at room temperature for 30 minutes. The absorbance of colored product was subsequently measured between 300-700 nm using a Scanning UV-VIS Spectrophotometer (Shimadsu, Japan).

2.10.2 Dose-response formation curcumin-iron complex

Effect of iron concentration

Curcumin solution (a final concentration of 1 mM in MOPS buffer at pH 7.0) was incubated with ferric nitrate or ferric citrate (20, 40, 80 and 100 μM at final concentrations) at room temperature for 30 minutes. Absorbance was measured spectrophotometrically as previously described in **Section 2.10.1.**

Effect of curcumin concentration

Curcumin solution (100, 200, 400 and 800 μM at final concentrations in MOPS buffer, pH7.0) was incubated with ferric nitrate or ferric citrate (a final concentration of 100 μM in MOPS buffer at pH 7.0) at room temperature for 30 minutes. Absorbance was measured spectrophotometrically as previously described in **Section 2.10.1.**

2.10.3 Time course of curcumin-iron complex formation *in vitro*

Curcumin solution (1 mM at a final concentration in MOPS buffer, pH7.0) was incubated with ferric nitrate or ferric citrate (20 and 80 μM at final concentrations in MOPS buffer, pH7.0) at room temperature for 0, 1, 3, 10, 20 and 30 minutes. Absorbance was measured spectrophotometrically as previously described in **Section 2.10.1.**

2.11 Efficacy of plasma NTBI removal by deferiprone and curcumin *in vitro*

Plasma of ESRD patients was divided into aliquots (0.55 ml each) and transferred to four separate sets. The first one (a control) was added with PBS and the latter was added with deferiprone solution (100 μM at in the presence of different curcumin concentrations (100, 200 and 400 μM). Tubes contents were incubated at 37 ^{o}C for exactly 1 hour. NTBI concentration was measured using the HPLC technique as previously described in **Section 2.7.**

2.12 Effect of curcumin on oxidative stress in whole blood

To determine the effect of curcumin on oxidative stress in peripheral whole blood, 0.9 ml of blood sample from of ESRD patients was first incubated with either PBS (as a control), curcumin (20 μM at a final concentration) or curcumin (100 μM at a final concentration) for 1 hour. Each tube contents were incubated with either PBS, 3%H_2O_2, Fe^{2+}-EDTA (300 μM iron concentration) or 3%H_2O_2 together with Fe^{2+}-EDTA (300 μM iron concentration) for 30 minutes, then diluted with Ca^{2+}-and Mg^{2+} - free phosphate buffered (PBS) to reach a concentration of 1x10^6 RBCs/ml. Then, DCFH-DA dissolved in methanol was added at 0.4 mM final concentration. After incubation at 37^{o}C for 15 minutes in a humidified atmosphere of 5% CO_2 in air, the RBC suspension was centrifuged at 4,000 rpm (1180g) at 10 ^{o}C for 10 minutes. The pellet was washed with PBS and resuspended in PBS. Then, RBC suspension was centrifuged at 4,000 rpm (1180xg) at 10^{o}C for 10 minutes. The pellet was washed twice with PBS and resuspended in PBS. Finally, RBCs were analyzed by a Fluoresence Actvated Cell Sorter a previously described in Section 2.8.1. A two-parameter dot-plot of the side light scatter (SSC) and forward light (FSC) of the population was first analyzed and FL-1 was included. The Geometric Mean fluorescence channel was derived by CellQuest® software.

2.13 Effect of curcumin on oxidative stress in RBC suspension

For determination of the effect of curcumin on oxidative stress in RBC, 2 μl of peripheral blood samples of ESRD patients was diluted with Ca^{2+}- and Mg^{2+}- free phosphaste buffered saline (PBS) to reach a concentration of 1x10^6 RBCs/ml. DCFH-DA, previously dissolved in methanol, was then added at a final concentration of 0.4 mM. After incubation at 37^{o}C for 15 minutes in a humidified atmosphere of 5% CO_2 in air, the RBC suspension was centrifuged at 4,000 rpm (1180xg) at 10 ^{o}C for 10 minutes. The RBC pellet was washed twice with PBS and resuspended in 2ml of PBS. Then, the RBC suspension was centrifuged at

4,000 rpm (1180x g) at 10°C for 10 minutes. The pellet was washed with and resuspended in 1 ml of PBS. The RBC suspension was divided into 0.45 ml-aliquots, which they were incubated with either PBS, curcumin (20 μM at a final concentration) or curcumin (100 μM at a final concentration) at room temperature for 30 minutes. The RBC suspension was further incubated with either 35 μl of the following solutions: PBS (as a control), 3%H_2O_2, Fe^{2+}-EDTA (300 μM at a final iron concentration) and 3%H_2O_2 together with Fe^{2+}-EDTA (300 μM at a final iron concentration) for 20 minutes. Finally, RBCs were analyzed by a Fluoresence Actvated Cell Sorter (FACSort, Becton Dickinson, Immunofluorometry systems, Mountain View, CA, USA) previously described in Section 2.8.1. A two-parameter dot-plot of the side light scatter (SSC) and forward light (FSC) of the population was first analyzed and FL-1 was included. The Geometric Mean fluorescence channel was derived by CellQuest® software.