CHAPTER IV

DISSCUSSION AND CONCLUSION

Anemia in ESRD patients on regular hemodialysis can be treated with the subcutaneous injection of recombinant human erythropoietin (rHuEPO) or with intermittent blood transfusion (Boran, *et al* 1993, Zehnder and Blumberg 1989). Sometimes, intravenous iron injection is available and necessary for the ESRD patients to improve their anemia as well. Intravenous iron is used to optimize a response to (rHuEPO) in ESRD patients (Van Wyck 1989). However, no consensus exists with respect to the best regimen to avoid transferrin oversaturation, oxidative stress and the occurrence of non-transferrin-bound iron (NTBI) in their blood circulation. It was found that ESRD patients, who they had received intermittent blood transfusion (ESRD-T) or rHuEPO concomitant with iron tablets (ESRD-E), were significantly anemic than normal healthy volunteers. Moreover, their plasma iron and transferrin saturation levels were more highly saturated when compared with the control. When the NTA chelation/HPLC-based assay was used to investigate plasma NTBI concentrations in these ESRD patients, the result demonstrated that plasma NTBI level in ESRD-T patients was significantly higher than ESRD-E patients ($p<0.05$). Because each human blood unit contains about 0.2 g of iron, the patients who received several blood transfusions become overloaded with iron leading to saturation of transferrin and often the appearance of NTBI in the blood. Regarding to plasma malondialdehyde (MDA), the most common indicator for monitoring lipid peroxidation (Bergamini, *et al* 2004), MDA level in ESRD-T patients was higher than ESRD-E insignificantly ($p=0.09$) (Table 3.4). Previous research has concentrated upon the HD group, where enhanced lipid peroxidation may be resulted from free radical activity generated by complement activation and released cytokines as well as from an exposure of blood to bio-incompatible dialysis memebranes during the dialysis procedure. It has been speculated that the transient presence of free iron, as it transits from its bound form to the reticuloendothelial system, may lead to increased reactive oxygen species (ROS) generation consequently production oxidative stress and subsequent atheromatous changes (Cheung 1994, Kolb, *et al* 1991, Wauters 1995). Lim and coworkers reported that hemodialysis patients presented a greater decrease in the plasma levels of superoxide dismutase (SOD) antioxidant enzyme and a greater increase in lipid peroxides after intravenous administration of 100 mg ferric saccharate (Lim, *et al* 1999).

Oxidative stress in RBCs of ESRD patients

Although ROS such as hydrogen peroxide and hypochlorite are normally secreted by neutrophilic leukocytes for killing microorganisms, they are also by-products of metabolism which can oxidize various molecules leading to cell death and tissue injury. ROS contribute to several pathogenesis. Iron overload status in ESRD patients is one of risk factors that can potentially increase free radical generation. In this study, we have used a powerful and sensitive flow cytometric technique to investigate the oxidative stress in red blood cells of ESRD patients compared to normal volunteers. Oxidation of dichlorofluorescein (DCF) fluorochrome by ROS produced the green fluorescence intensity and the results were shown in Figure 3.5 A and 3.5B. The fluorescence intensity of H_2O_2-stimulated red blood cells was increased when compared with unstimulated red blood cells. Interestingly, the fluorescence intensity was higher in ESRD-E red blood cells than in ESRD-T red blood cells. It is possible that ESRD-E patients may be hyporesponsive to erythropoietin administration and have functional iron deficiency (Tarng, *et al* 1999). They therefore have apparently insufficient available iron to keep up with demands of the stimulated erythropoiesis that occur when exogenous erythropoietin is administered. This meant that iron store in ESRD-E patients can increase the ROS generation more than the other group. However, oxidative stress may be one of the important complications occurring in both groups. The multifactor nature of this process might include other factors such as the lack of complete correction of the uremia toxicity, malnutrition and the progressive worsening of the clinical condition due to aging and co-morbidity (Danielski, *et al* 2003, Kalantar-Zadeh, *et al* 2003, Odani, *et al* 1999). Altogether these factors trigger oxidative stress by establishing a steadily abnormal production of pro-oxidant stimuli coupled with a defective or insufficient antioxidant protection (Canaud, *et al* 1999, Vaziri, *et al* 2002).

Effects of curcumin on oxidative stress

The effect of curcumin on oxidative stress using the flow cytometry was examined in whole blood and red blood cell suspension of ESRD patients that were untreated and treated with Fe^{2+}-EDTA. Using whole blood in the presence of H_2O_2 it was found that curcumin at concentrations of 20 and 100 μM did not affect or decrease the ROS level when compared to PBS-treated red blood cells as a control. While using red cell suspension, curcumin at 100 μM was able to decrease the ROS level considerably (p=0.05) when compared to the control cells. Therefore, the free-radical scavenging activity of curcumin was more effective in red cell suspension than in whole blood. Probably, whole blood itself contains many anti-

oxidants such as proteins (especially albumin), uric acid, bilirubin and creatinine in the plasma compartment, which these compounds can counteract the activity of free radicals immediately(Malliaraki, *et al* 2003, Mancuso, *et al* 2003, Waring, *et al* 2003). ROS production was slightly increased in the ESRD red blood cells treated with Fe^{2+}-EDTA. Previously mentioned, iron catalysed the reduction of molecular oxygen in water to sequentially form products including superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in Haber-Weiss reaction and the breakdown of hydrogen peroxide to hydroxyl radical ($HO^\bullet$) in Fenton reaction (Pippard 1999). However curcumin can decrease ROS production because it is capable of scavenging oxygen free radicals such as superoxide anions and hydroxyl radicals. The phenolic and the methoxy group on the phenyl ring and the 1,3-diketone system seems to be important structural moieties that can contribute to these effects (Patro, *et al* 2002).

Effect of *in vivo* hemodialysis on plasma NTBI level

From the study of *in vivo* hemodialysis on plasma NTBI level in four ESRD patients, the result reveals that after hemodialysis plasma NTBI values in all four patients were decreased from predialysis values slightly but insignificantly ($p = 0.63$) (Figure 3.10) (Table 3.7). This suggests that hemodialysis process can not only get rid of excess waste products like ammonia, urea, creatinine and phosphate but also reduce some NTBI in plasma of these ESRD patients. Although plasma NTBI is potentially a harmful iron and can participate in free-radical production, the nature of NTBI has not been exactly identified but postulated as low and high MW forms(Esposito, *et al* 2002, Hider 2002). Low MW NTBI is chelatable or accessible iron bound to citrate, phosphate, oxalate and some amino acids. High MW NTBI is unchelatable or inaccessible iron loosely bound to other ligands such as plasma proteins (especially albumin). Under hemodialysis process, low MW NTBI can pass through dialysis membrane and passively diffuse into the dialysate solution gradually. Nevertheless, some of NTBI remained in the plasma after 4-hour hemodialysis, which it was presumed as the high MW NTBI.

Effect of *in vitro* hemodialysis on plasma NTBI level

As mentioned above, *in vivo* hemodialysis had decreased NTBI level in blood of ESRD patients. Similarly, *in vitro* hemodialysis alone had decreased plasma NTBI likely due to concentration-gradient passive diffusion. In comparison, removal of plasma NTBI by deferiprone was more rapid but remained incomplete. In this study, curcumin at a concentration of 800 μM together with 100 μM deferiprone

decreased 22 % NTBI when compared to the control. This suggests that DFP can promote or accelerate the mobilization of plasma NTBI, mostly the low MW NTBI and partially the high MW NTBI. In addition, curcumin had a synergistic effect with deferiprone to remove NTBI from blood of ESRD patients. Previous report showed that β-diketo moiety of curcumin has a strong ability to chelate metal ions while the methoxy group on the phenyl ring of curcumin can scavenge oxygen free radicals (Patro, *et al* 2002). This synergistic effect can not only remove excess free plasma iron as NTBI from the body, but also be used in the detoxification free radical in ESRD patients.

Effect of curcumin on the *in vitro* removal of NTBI by deferiprone

Curcumin and its derivatives have shown the ability of being free-radical scavenger, interacting with oxidative cascade, quenching oxygen and chelating and disarming oxidative stress properties of metal ions (Masuda, *et al* 1999, Patro, *et al* 2002).

Curcumin bound ferric ion (Fe-nitrate, Fe-citrate) in the form of ferric-curcumin complex, which was a chromophore exhibiting the absorption at 500 nm. This chemical iron binding onto curcumin was rapid and concentration-dependent (Figure 3.13, Figure 3.14). Spectrophotometric analysis indicated the *in vitro* interaction of ferric ion was not only concentration-but also time-dependent. Compatible to the previous study found that biological activity of curcumin has been attributed to the hydroxyl group substituted on the benzene rings and also to the diketonic structure. The β-diketo moiety of curcumin undergoes a keto-enol tautomerisation. Crystalographic studies have shown that the symmetric structure of curcumin leads to statistically even distribution of the enol proton between the two oxygen atoms. The strong chelating ability of diketones has been widely investigated to a great number of metal ions; therefore, curcumin could be of great importance in the chelating treatment of metal intoxication and overload.

In addition to HPLC- based method was used to investigate the effect of curcumin and deferiprone to removal plasma NTBI. The results reveal that in the presence of deferiprone at 100 μM, curcumin can reduce plasma NTBI level of ESRD with concentration dependent. The curcumin at 400 μM with 100 μM deferiprone had decreased NTBI with more than 50%.

Apparently, NTBI was detectable in some ESRD patients and partially lost during *in vitro* and *in vivo* hemodialysis. Further characterization of native form of NTBI in ESRD plasma is recommended. It needs to investigate the NTBI remaining after such hemodialysis whether it is still harmful to the biomolecules and tissues or not. Larger numbers of NTBI-positive ESRD plasma are necessary to clarify the efficacy of

hemodialysis, curcumin and deferiprone administration. Because curcumin cooperated with deferiprone to remove plasma NTBI effectively in ESRD patients *in vitro*, it could be used for clinical trials in these patients with precautions. Effective and safe dose of curcumin has to be optimized in order to relieve or prevent the oxidative stress in red blood cell compartment of ESRD patients.

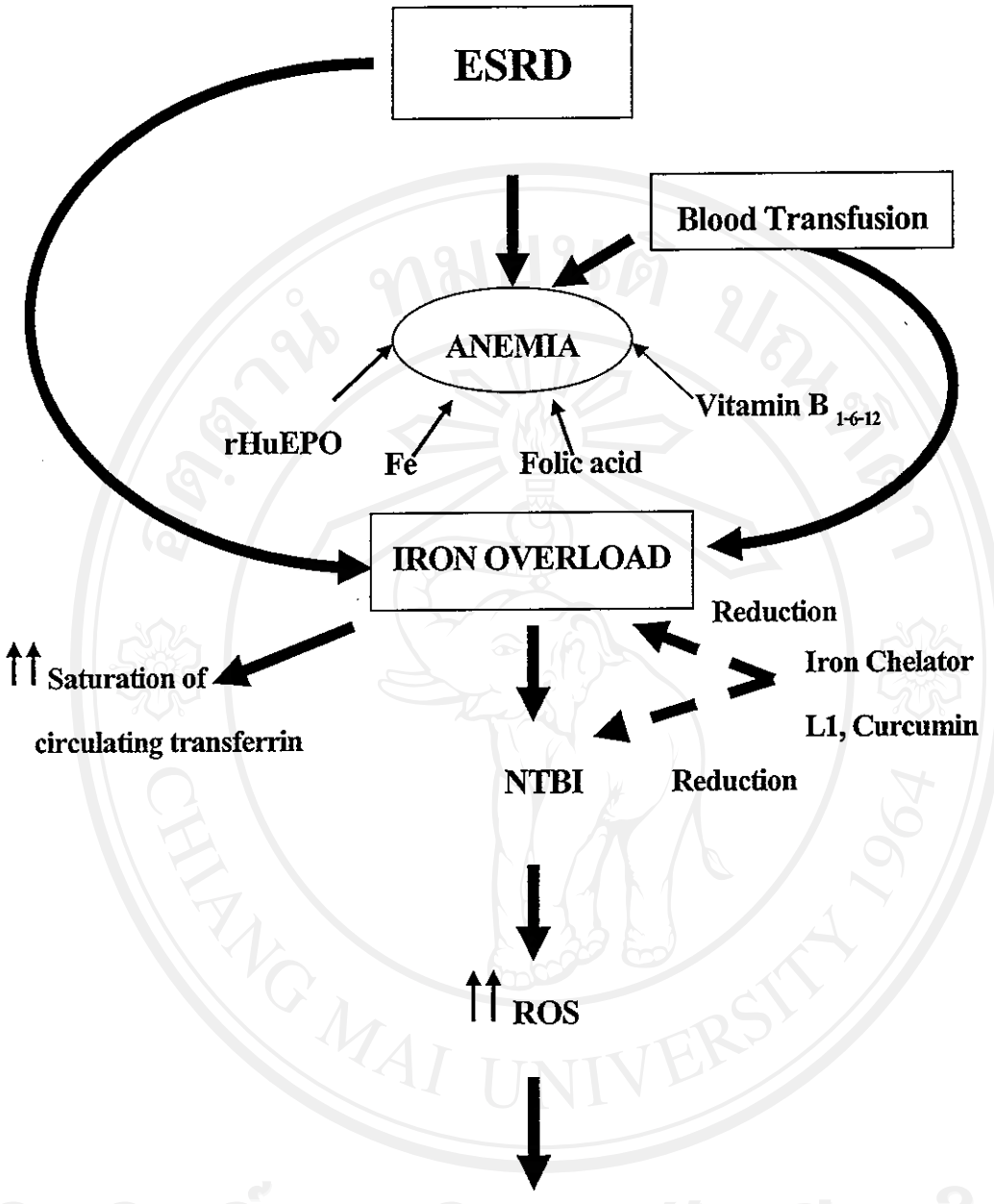

Figure 4.1 Overview of iron overload and oxidative stress in ESRD patients on regular hemodialysis (↑↑ = increase).