II. MATERIALS AND METHODS

A. Materials

1. Subjects

Normal control samples include normal cord blood from the Delivery unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University. All have normal hematological indices. Post splenectomy thalassemic patients were recruited from Department of Pediatrics, Faculty of Medicine, Chiang Mai University.

About 20 mL of whole blood of β-thalassemic patients and normal cord blood were collected in acid citrated dextrose (ACD) ratio 9:1.

B. Methods

PART I.

Determination of NRBCs and PBMCs apoptosis in β-thalassemic patients.

1. **Determinations of the complete blood cell count and hemoglobin typing**

Complete blood cell count was analyzed by automated cell counter (Hycell Groope Lisabio) and hemoglobin typing was analyzed by Primus Variant HPLC Analyzer (Primus Corporation).

2. **Nucleated red blood cells (NRBCs) and peripheral blood mononuclear cells (PBMCs) preparation**

Nucleated red blood cells and peripheral blood mononuclear cells were isolated by centrifugation in Ficoll-Angiografin density gradient (1.077) at 400g for 10 minutes. The NRBCs and PBMCs were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and about 100,000 units of penicillin and 0.1 g of streptomycin. After cells were counted, they were stained with Wright's Giemsa stain and observed for purity and with trypan blue stained for viability. The NRBC and PBMC should be 90% or more in cell suspension.

3. **Examination of Comet assay**

The single cell gel electrophoresis (SCG) assay or comet assay was modified from Gopalakrishna P and Khai A, *et al.*, 1995, Henderson L, *et al.*, 1998, Florent M, *et al.*,1999, Puaninta C, 2001 and Benjamin E, *et al.*, 2001.

3.1 **Preparation of slides for the single cell gel assay**

3.1.1 0.75% Low melting point agarose (LMA, 375 mg per 50 mL) and 1.0% normal melting agarose (NMA, 500 mg per 50 mL) were prepared in PBS. The NMA (Sigma A6877) was dissolved in PBS by microwave or heat until near boiling. The LMPA (Sigma 40K0608) were kept in aliquot 5 mL samples into scintillation vials (or other suitable containers) and refrigerate until needed. When needed, briefly melt agarose in microwave or hot plate. LMPA vial was placed in a 37°C water bath.

3.1.2 While NMA agarose was heated, fully frosted slide 25.4x76.2 mm. were precoated with 90 μL of 1% NMA agarose, covered with a cover slip 22x26 mm. The slide was laid on a flat surface in a tray and placed in the 4°C refrigerator for 10 minutes, then cover slip was removed.

3.1.3 To the coated slide 90 μL of LMPA was added and mixed with 10μL containing 10,000 cells approximated NRBC or PBMC. The coverslip was replaced by a surgical blade (Razor Co., Ltd) and the slide was placed in a slide tray at 4°C for 10 minutes until the agarose layer stucked to the slides.

3.1.4 The coverslip was removed and the slide was lowered in cooled down, freshly made lysing solution kept refrigerated in the dark (protected from light and refrigerated) for at least 1 hour. The, slides may be stored for at least 4 weeks in cold lysing solution without affecting.

NOTE: If the gels were not stuck to the slides properly, avoiding humidity and/or increasing the concentration of NMA agarose in the lower layer to 1.5% will eliminate the problem. Step 3.1.3 to 3.1.4 should be performed under dim yellow lights to avoid DNA damage.

3.2 **Electrophoresis of microgel slides**

3.2.1 After at least 1 hour standing slides at about 4°C, the slides were removed gently from the lysing solution. The slides were rinsed carefully in Tris [Hydroxymethyl] aminomethane (e.g. 3 - 5 minutes) to remove detergents and salts. The slides were placed side by side as close together as possible on the horizontal gel box near the cathode.

3.2.2 The buffer reservoirs were filled with freshly electrophoresis buffer pH 13 until the liquid level completely covered the slide (avoid bubbles over the agarose).

3.2.3 Slides were allowed in the alkaline buffer for 20 minutes for unwinding of the DNA and the expression of alkali-labile damage. The longer the exposure to alkali, the greater was the expression of alkali-labile damage.

3.2.4 The power supply 25 volts with the current to 300 milliamperes was adjusted by raising or lowering the buffer level. The slides were electrophoreses for 20 minutes.

3.2.5 The power was turned off. The slides were gently lifted from the buffer and placed on a drain tray. Dropwise the slides were coated with neutralization buffer, let there allowed to stand at least 5 minutes

3.2.6 Slides were stained with 20 μL 1X ethidium bromide (20 μg/mL) and immediately scored.

3.3 **Evaluation of DNA damage**

3.3.1 For visualization of DNA damage, after fluorochrome-stained DNA, the DNA damage were observed under a 100 x objective on a fluorescent microscope.

3.3.2 The Comet analysis system used in this study developed by kinetic imaging, Zeiss Microscope model axioskop 2 both fluorescent and tungsten light function linked to a charge-coupled device camera (CCD camera) to quantify the length of DNA migration and percentage of migrated DNA. To distinguish between populations of cells differing in size (e.g. PBMC *versus* NRBC), we also measured nuclear diameter. Finally, the ISIS3 comet image program calculates comet tail moment (*mt*). Generally, 30 randomly selected cells were analyzed per sample.

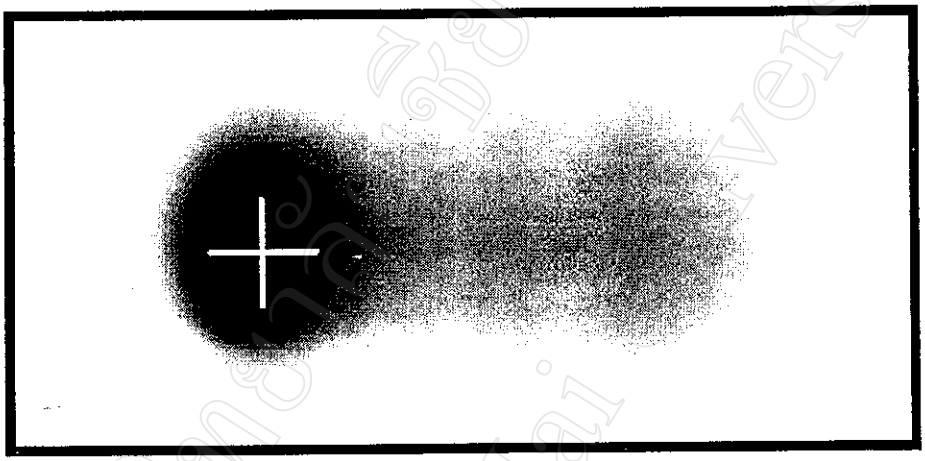
3.3.3 Statistical analysis for *in vitro* data were one-way ANOVA, independent-sample t test and correlation, we analyzed the number of cells which exhibit values greater than the 95 or 99% confidence limits for the distribution of control data (i.e., the frequency of damaged *versus* undamaged cells).

The parameters were calculated according to the formula:

$$mt = Xm \bullet Ft$$

$$Xm = [\Sigma_t (Ii \bullet Xi)] / (\Sigma_t Ii)$$

$$Ft = (\Sigma_t Ii) / \Sigma_c Ii)$$



Comet tail moment (*mt*) and the product of DNA portion in the comet tail (*Ft*) and its median (*Xm*). Where *Ii* was fluorescence intensity at point *i* , and *Xi* was the distance from the comet head median to point *i.* Indices under the sum symbols imply the summation region : *t*, the summation is over the comet tail only; *c*, the summation is over the whole comet.

PART II

Factor Affecting Apoptosis

1. An effect of β-thalassemic plasma on cell oxidatively stressed

An effect of β-thalassemic plasma on cell oxidatively stressed was modified from Banjerdpongchai R, *et al.* 1997. PBMCs from normal cord blood and patient were analyzed by treating the cells with β-thalassemic plasma (v/v). The cell were prepared at $1.0x10^6$ cells/mL suspension in the RPMI-1640 with 10% fetal calf serum (FCS), penicillin G (100 units/mL) and streptomycin (100 μg/ml) on 24 well culture cluster (Corning Incorporation). The cells were cultured for 12 and 24 hours at 37°C in 5% CO_2.

β-Thalassemic plasma was added to the supplement media instead of fetal calf serum. The concentrations of the plasma were 10, 20, 30, 40 and 50 % respectively. And the dose response was studied. After adding β-thalassemic plasma, the 24 well plate was incubated at 37°C in 5% CO_2. After incubation, the cells were analyzed by comet assay.

2. The effect of iron and H_2O_2 on cells oxidatively stressed and apoptosis

The iron was stored in cell as low molecular weight iron chelates, heme associated iron, ferritin bound iron in the form of Fe^{3+}, and non-transferin iron. The leakage of the red cell contents such as Fe^{2+} and hemin has been found to induce the oxidative damage or trigger reactive oxygen species (ROS) production (Anderson D, *et al.*, 2000)

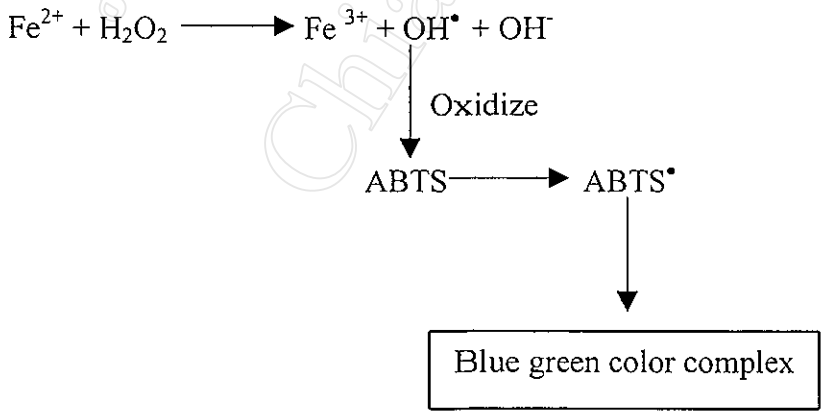
The role of Fe^{2+} on oxidant injury to the NRBC was intriguing since Fe^{2+} was a catalyst capable of simulating ROS generation, e.g. $OH^{\bullet}$.

Hydrogrn peroxide could react with transition-metal catalysts to generate the hydroxyl radical ($OH^{\bullet}$). Fenton reaction in 1894 as shows below basically described the sequence of the reactions:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3-} + OH^{\bullet} + OH^{-}$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + O_2^{\bullet} + 2H^{+}$$

This so-called iron-catalyzed Haber-Weiss reaction, was first proposed as one of several possible reaction (Halliwell B and Gutteridge JMC, 1989). In this study we used Fenton reacton produced hydroxyl free radical then ROS production would be induce oxidative stress to cell. The Fenton reaction was modified from Suttajit M, *et al.*, (2000).



Fe^{2+} was reacted with H_2O_2 and produced hydroxyl free radical. Later, hydroxyl free radical oxidized ABTS, and produced the blue green color complex that could be measured at absorbance 414 nm.

2.1 **Titration of Fenton reaction**

$FeSO_4$ at 0.5, 1.0, 1.5 and 2.0 μM were used to find optimum concentration. PBS pH 7.4, 200 μL ABTS 20 μM and 500 μM H_2O_2 were fixed condition in this method.

First step, 790 μL of PBS pH 7.4, 200 μL of ABTS concentration 20 μM, $FeSO_4$ at varied concentration were added in a cuvette respectively, mixed well for 10 seconds. Then 1000 μL of 500 μM H_2O_2 was added in a cuvette, mixed well for 5 seconds

The reaction was measured by UV-240 PC spectrophotometer (Shimasu Co., Ltd). Acquisition rate was 5 seconds and reaction time was 60 second. The Fenton reaction curve was plotted on x-y scatter with absorbance 414 on the Y-axis and time (seconds) on the X-axis.

2.2 **Cytotoxicity test (Trypan Blue Exclusion)**

Determination cytotoxic of the $FeSO_4$ and H_2O_2 to cells was modified from Limtrakul P, *et al.*, 1997.

PBMCs of β-thalassemic patients and normal cord blood were prepared at 1.0 x 10^6 cells/mL suspension in the RPMI-1640 with 10% fetal calf serum (FCS), penicillin G (100 units/mL) and streptomycin (100 ug/ml) on 24 well culture cluster (Corning Incorporation).

The cells were treated with final concentration of 0.25, 0.5, 1.0 and 5.0 mM $FeSO_4$ in RPMI -1640 with10% FCS, 37°C in 5% CO_2 for 24 hours. Each cell types was also treated with 25, 50, 100, 250 and 500 μM H_2O_2 in RPMI -1640 with 10% FCS, 37°C in 5% CO_2 for 24 hours.

After cells were treated for 24 hours, they were washed twice with 10 mL of incomplete RPMI-1640 medium and centrifuged for 10 minutes at 2000 rpm. The pellet was resuspended in PBS buffer (pH 7.2) to a dilution of 10^5 cells/mL.

Then, 90 μL of cell suspension was mixed with 10 μL 5% trypan blue and stained for 5 minutes at room temperature. Twenty five μL of the cell sample was pipetted on microscope slide and covered with a coverslip, then observed by using microscope 400x. The living cells were noticed by exclusion of trypan blue and were counted as %.

2.3 **The effect of Fenton reaction on cell oxidatively stressed**

The cells were prepared at 1.0×10^6 cells/ mL suspensions in modified G-buffer pH 7.2. The cells were treated with $FeSO_4$ at concentration of 0.25, 0.5, 1.0 mM and added to H_2O_2 at concentration 25, 50, 100 μM. The reaction was incubated for 4 hours at 37°C in 5% CO_2. After incubation, the cells were washed twice with PBS pH 7.2 and centrifuged at 2000 rpm for 10 minutes. The pellet was resuspended in PBS for comet assay.

3. **The effect of curcumin on the inhibition of oxidative stress and apoptosis**

3.1 **A curcumin extraction**

Fifteen grams of turmeric powder was successively extracted with 100 mL of hexene at room temperature; oil was removed in this step. Defatted turmeric powder was extracted with absolute ethanol with 100 mL in round bottom flask at room temperature. The ethanolic extracted was filtered with Whatman No.1 for removing the remaining powder. The ethanolic extracted was evaporated at 60 °C 1-2 hours to concentrate the turmeric solution.

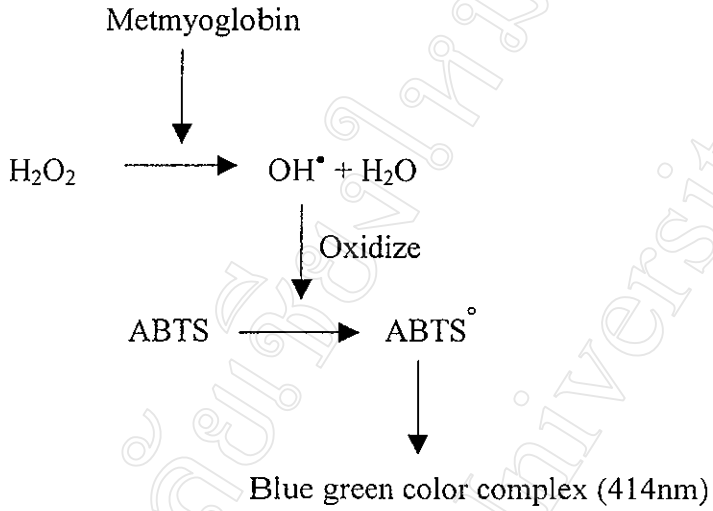
Curcumin should be kept at -20°C for 1 year and can be dissolved in DMSO or absolute ethanol for 1 month at 4°C.

3.2 **Purification of curcumin by thin layer chromatography (TLC)**

Curcumin was tested the purity by thin layer chromatography (TLC) compared with commercial curcumin (curcumin: demethoxycurcumin: bisdemethoxycurcumin ; 79:19:2, ICN, USA). The aluminum-coated silica gel was used as the stationary phase and mobile phase was the mixed solution of chloroform: absolute ethanol: acetic acid (94: 5: 1, v/v).

The curcumin extract was spotted on silica gel (5 x 8cm) diameter 2 mm at a distance 1 cm from the bottom of gel. The silica gel was dipped in chamber of mobile phase for 30 minutes, and then distance of the sample and mobile phase were measured for calculation the rate of flow (RF).

3.3 **Reactive oxygen species scavenger activity of the extracted curcumin**



Metmyoglobin reacted with H_2O_2 and produced hydroxyl free radical. Later, hydroxyl free radical oxidized ABTS occurred the blue green color complex can be measured at absorbance 414 nm.

First step, the reaction was done by added 1000 μL of PBS (pH 7.4), 50 μL of metmyoglobin concentration 400 μM and 500 μL of ABTS concentration 500 μM respectively, mixed well for 10 seconds. Then H_2O_2 concentration 500 μM 450 μL was added in a mixture, mixed well for 5 seconds.

The reaction was measured by UV-2401 PC spectrophotometer (Shimasu Co., Ltd. Acquisition rate 5 seconds, reaction time 180 seconds. The negative control curve was plotted on x-y scatter with absorbance 414nm on the Y-axis and time (seconds) on the X-axis.

Seconds, curcuminoid at various concentration were added in reaction for inhibit oxidation reaction. As well as the reaction was measured by UV-2401 PC spectrophotometer. The curve were plotted on x-y scatter with absorbance 414 nm on the Y-axis and time (seconds) on the X-axis. Then calculated % inhibition of oxidation from formula:

$$\% \text{ inhibition} = \frac{[A\ 180(\text{negative control}) - A\ 180(\text{test})]}{A\ 180(\text{negative control})} \times 100$$

In this study Trolox was used to be a reference for measured % inhibition of oxidation. % Inhibition oxidation of Trolox was modified from Suttajit M, *et al.*, 1997. The concentrations of Trolox 4, 5, 6, 8, 10 and 13 μg/mL were used for standard curve of Trolox in this study.

ABTS method was prepared by 50 μL of 400 μM metmyoglobin, 1000 μL of PBS pH 7.4, 500 μL of 500 μM ABTS were added in a cuvette respectively, mixed well for 5 seconds and followed with 450 μL of 500 μM H_2O_2 mixed well for 5 seconds, measured absorbance at 414 nm. The first curve was negative control. Later, Trolox at vary concentration were added in the reaction. The reaction was measured by UV-2401 PC spectrophotometer. Acquisitions rate 5 seconds and reaction time 180 seconds.

The stand curve of Trolox was plotted on x-y scatter with % inhibition on the Y-axis and concentration (μg) on the X-axis.

3.4 **Cytotoxicity test by trypan blue exclusion**

Cytotoxicity of curcuminoid to cell was modified by Limtrakul P, *et al.*, (1997). Normal PBMC were prepared at the concentration of $1.0x10^6$ cells/mL in completed RPMI-1640 medium and then cells were treated with 1, 5, 10, 15 and 20 μg/mL curcumin at 37°C, in 5% CO_2 for 12 hours.

After cells were treated for 12 hours, they were washed with incompleted RPMI-1640 medium for 2 times and then centrifuged at 2000 rpm for 10 minutes. The pellet was resuspended in PBS buffer (pH 7.2) and prepared a dilution of 10^5 cells/mL.

Then, 90 μL of cell suspension was determined by 10 μL 5% trypan blue and stained for 5 minutes at room temperature. Twenty five μL of the cell sample was pipetted on microscope slide and covered with a coverslip, then observed by using microscope 400x. The cell viability was noticed by exclusion of trypan blue and counted as the present of a hundred total cells.

3.5 **Effect of curcumin treatment on apoptosis inhibition**

Normal PBMCs at $1.0x10^6$ cells/mL suspension in completed RPMI-1640 medium were treated with curcumin concentration of 5, 10, 15 and 20 μg/mL 37°C, 5% CO_2 for 30, 60, 90 and 120 minutes. After incubation, the cells were washed twice with incomplete RPMI-1640 and centrifuged at 2000 rpm for 10 minutes. The cell pellets were resuspended in 1 mL of PBS pH 7.2, then the cells were treated by Fenton reaction as in 2.3. After Fenton reaction, DNA damages were analyzed by comet assay. Cell suspensions were also cotreated with Fenton reaction at 5, 10, 15 and 20 μg/mL curcumin. After Fenton reaction, cells were analyzed by comet assay.