III. RESULTS

PART I

1. Complete blood cell counts and hemoglobin typing

Two groups of samples were studied sequentially. Group 1 thirty post splenectomied β-thalassemic patients were selected. Eleven male and 19 female patients with transfusion-dependent β-thalassemia major, age ranging from 4 to 15 years. All patients were chronically transfused. The types of hemoglobin were FA and EF (25 and 5, respectively).

Nucleated red blood cell (NRBC) and peripheral blood mononuclear cell (PBMC) were counted by automated cell counter. The absolute number of NRBC and PBMC were 20.82 ± 27.97 x 10^6 cells /μL and 4.13 ± 1.74 x 10^3 cells/μL, respectively. Average mean ± SD of hemoglobin concentration, hematocrit and red blood cell count were 5.84 ± 1.54 g/dL, 19.56 ± 4.7 % and 2.56 ± 0.65 x 10^6 cells/μL, respectively.

Groups 2, five normal cord blood were selected from the Delivery unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University. All had normal hematological indices and used as control group.

The absolute number of NRBC and PBMC were 1.72 ± 0.54 x 10^6 cells/μL and 5.02 ± 0.87 x 10^3 cells/μL, respectively. Average mean ± SD of hemoglobin concentration, hematocrit and red blood cell count in mean ± SD were 12.89 ± 1.33 g/dL, 42.36 ± 3.13 % and 4.11 ± 0.49 x 10^6 cells/μL, respectively.

An increase absolute number of NRBCs was also observed in thalassemic patients compared with normal cord blood ($P<0.05$).

Table 3. Hemoglobin concentration of β-thalassemic patients (n=30)

Hb (g/dL)	0-4	5-10	>10
Number (30)	9	20	1
Percentage (%)	30	66	4

Hemoglobin concentration less than 10 g/dL was found in 96 % (n=30) of the β-thalassemic patients. Result showed that most of the patients were anemia as shown in Table 3.

Table 4. Hematocrit of β-thalassemic patients (n=30)

Hematocrit (%)	<15	15-30	>30
Number (30)	5	24	1
Percentage (%)	16	80	4

Hematocrit less than 30% was found in 96% (n=30) of the β-thalassemic patients. Table 4, hematocrit was responsible with hemoglobin concentration.

Table 5. Red blood cell count of β-thalassemic patient (n=30)

RBC (10^6 cells/μL)	<3	3-5	>5
Number (30)	23	7	0
Percentage (%)	76	24	0

Red blood cell count less than $3x10^6$ cells/μL was found in 76 % (n=30) of β-thalassemic patient as shown in Table 5.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

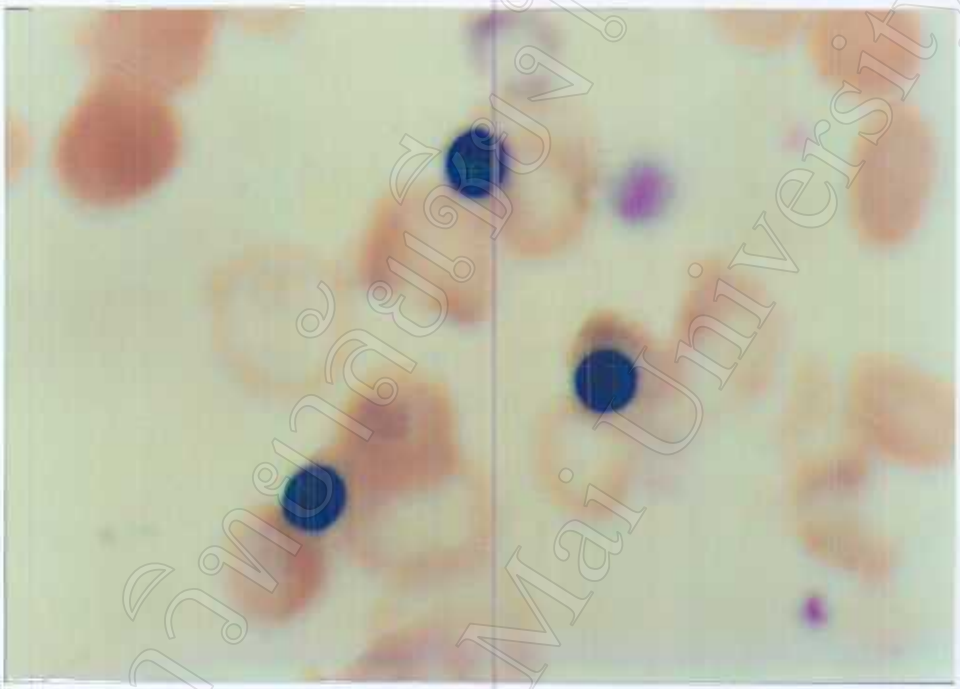

Figure 7. NRBCs of β-thlassemic patient

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

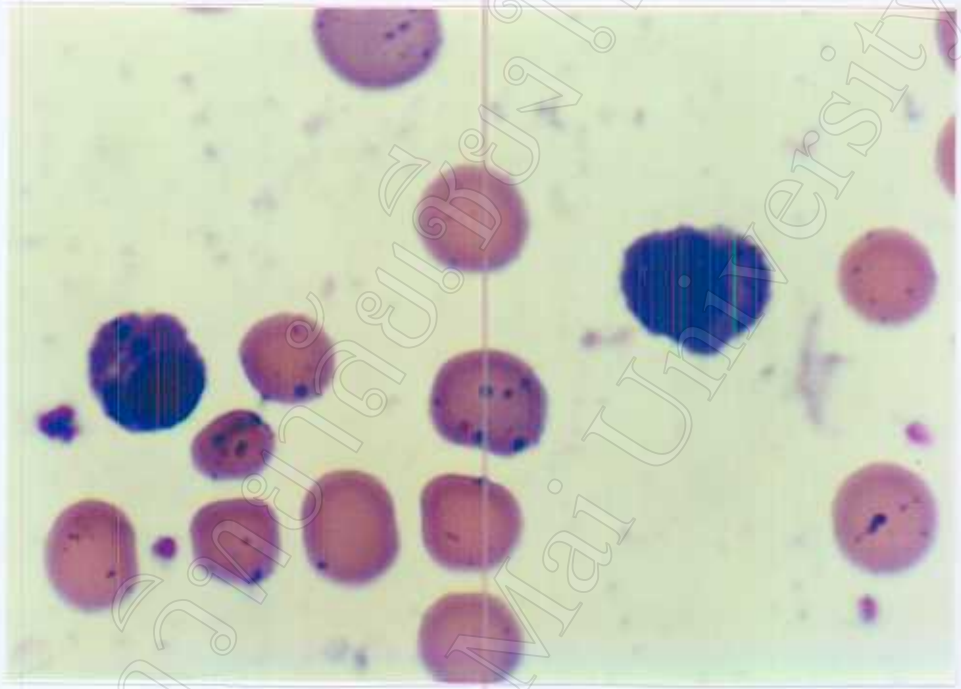

Figure 8. NRBCs of normal cord blood

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

2. **Apoptotic cells by comet assay**

NRBC and PBMC of β-thalassemic patients were measured apoptosis in individual cells by comet assay. Comet tail moment of NRBC more than 500 pixels was found in 86% (n=30) of β-thalassemic patients. The high value suggested that NRBC of β-thalassemic patients had a lot of DNA strand break as shown in Table 6.

Table 6. Comet tail moment (mt) of NRBC β-thalassemic patients (n=30)

Comet tail moment (pixels)	480-500	500-520	>520
Number (30)	4	13	13
Percentage (%)	14	43	43

มหาวิทยาลัยเชียงใหม่ Chiang Mai University

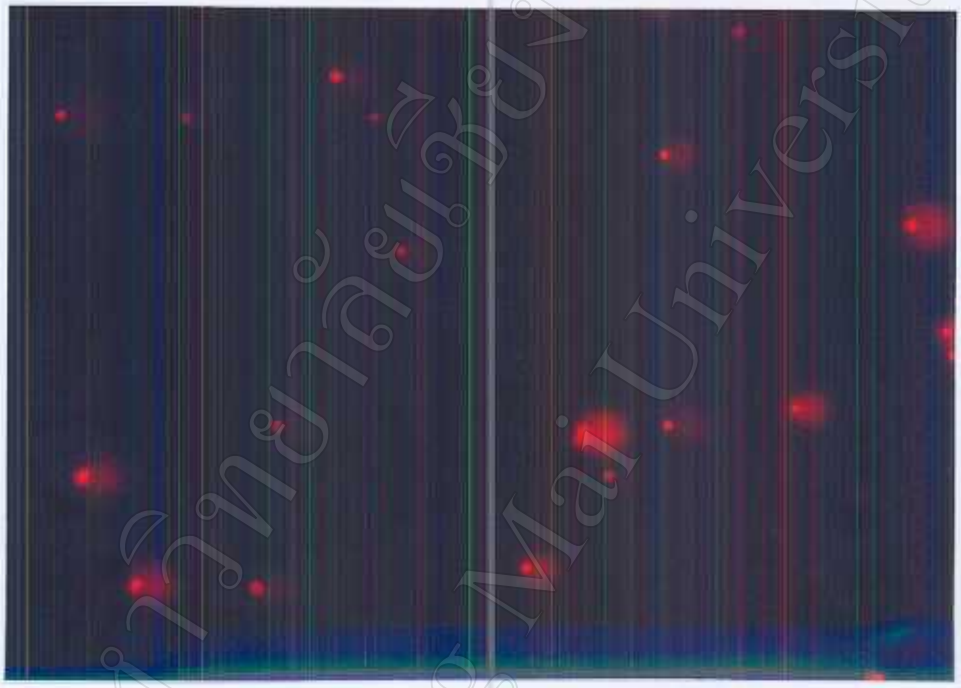

Figure 9. Comet cells (Apoptotic NRBC of β-thalassemic patients): 160X

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

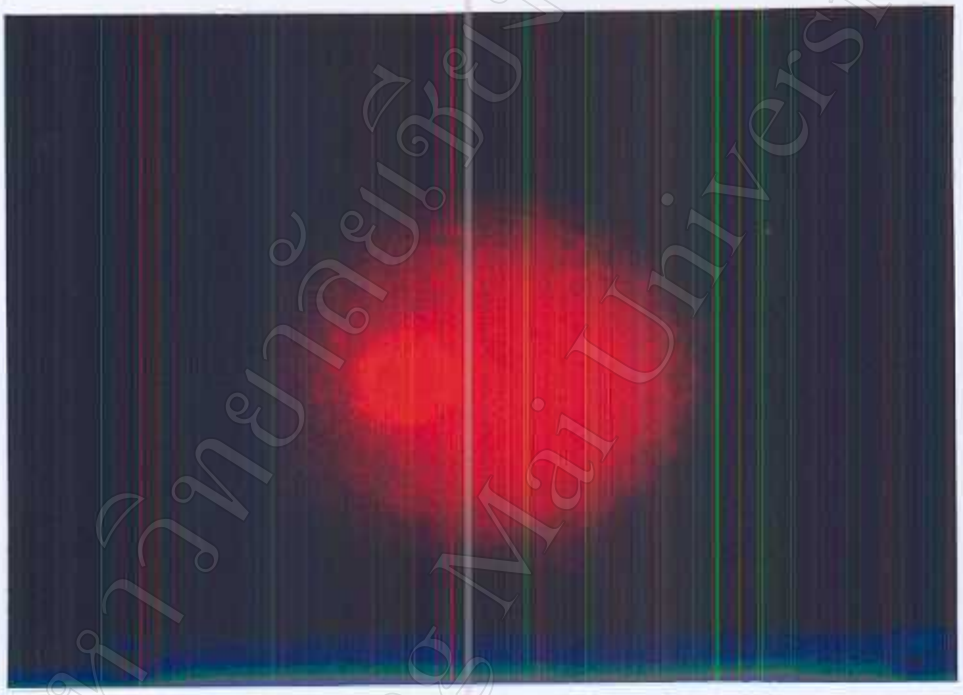

Figure 10. Comet cell (Apoptotic NRBC of β-thalassemic patients): 1000X

มหาวิทยาลัยเชียงใหม่ Chiang Mai University

Comet tail moment of PBMC more than 100 pixels was found in 83% (n=30) of β-thalassemic patients. The high value suggested that PBMC of β-thalassemic patients had DNA damage as shown in Table 7.

Table 7. Comet tail moment (mt) of PBMC β-thalassemic patients (n=30)

Comet tail moment (pixels)	<100	100-200	>200
Number (30)	5	25	0
Percentage (%)	17	83	0

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

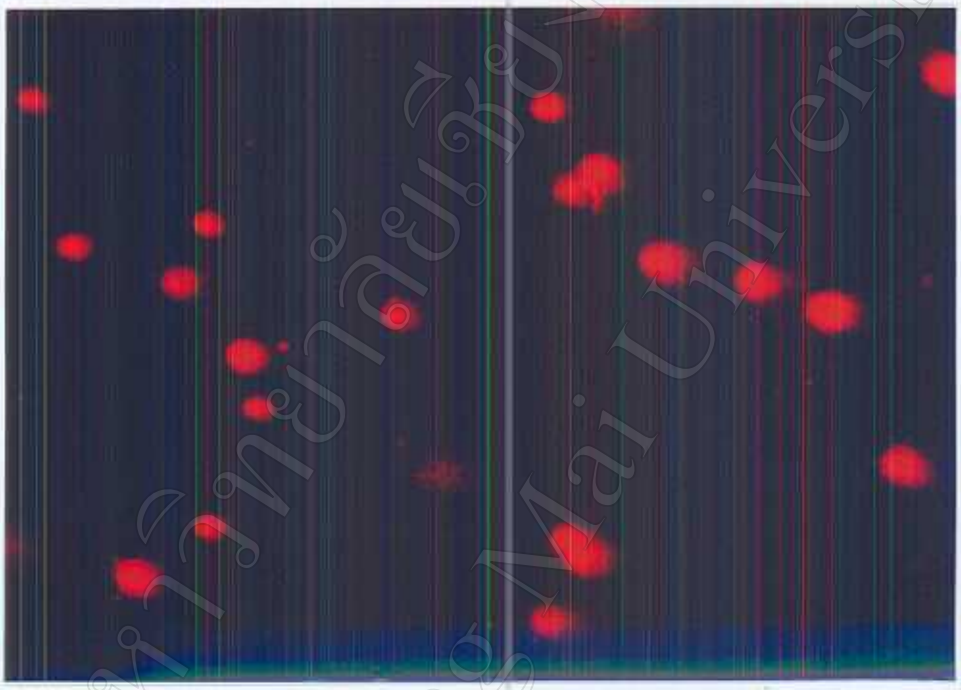

Figure 11. Comet cells of PBMC β-thalassemic patient with 160X

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

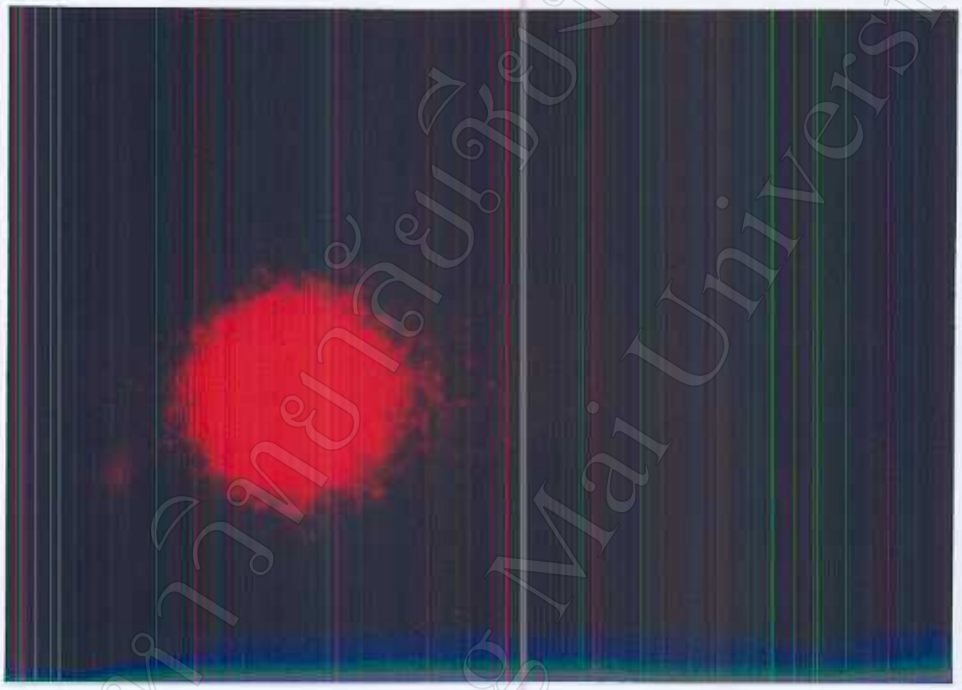

Figure 12. Comet cell of PBMC β-thalassemic patient with 1000x

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

PART II

1. Correlation of hemoglobin and comet tail moment of NRBC in β-thalassemic patients

An important consideration was whether severe anemia was characterized by increased NRBC apoptosis. Then correlation of the hematological indices and comet tail moment was studied and found that hemoglobin and comet tail moment of NRBC β-thalassemic patients were (mean ± SD) 5.84 ± 1.51 g/dL and 515.70 ± 12.10 pixels respectively ($r = -0.15$, $p = 0.5$) as shown in Figure 9. A decreased hemoglobin was also observed in thalassemic patients compared with normal cord blood (5.84 ± 1.54 *versus* 12.89 ± 1.33 g/dL, $p < 0.05$). Hemoglobin had a lower value than normal (range 12 to 15 g/dL) which suggested the patients had severe anemia. The high comet tail moment value that indicated a lot of DNA strand break of NRBC.

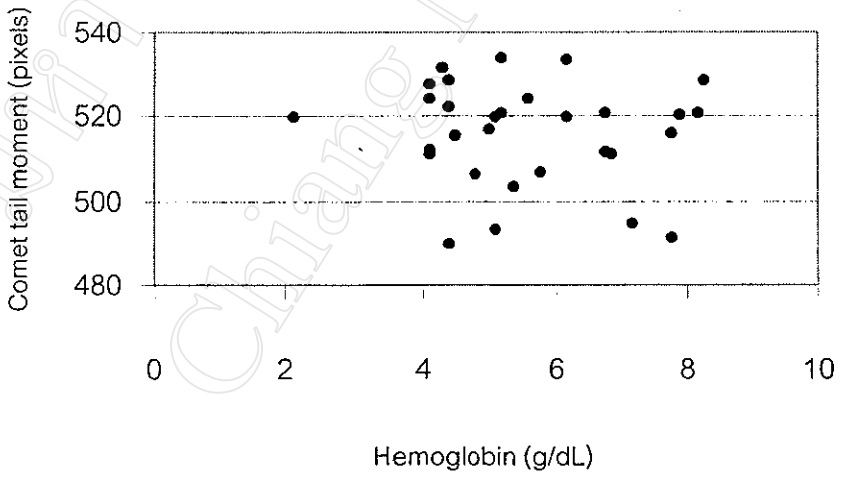

Figure 13. Correlation of hemoglobin and comet tail moment of NRBC in β thalassemic patients (n=30).

2. **Correlation of hematocrit and comet tail moment of NRBC β-thalassemic patients**

Hematocrit and comet tail moment of NRBC β-thalassemic patients (mean ± SD) were 19.56 ± 4.7 % and 515.70 ± 12.10 pixels, respectively, (r = -0.01, p = 0.05) as shown in Figure 10. Hematocrit had a lower value than normal range (35-40 %). A decreased hematocrit was also observed in thalassemic patients compared with normal cord blood (19.56 ± 4.7 *versus* 42.3 ± 3.13 %, $p< 0.05$). The high comet tail moment value also indicated a lot of DNA strand break of NRBC.

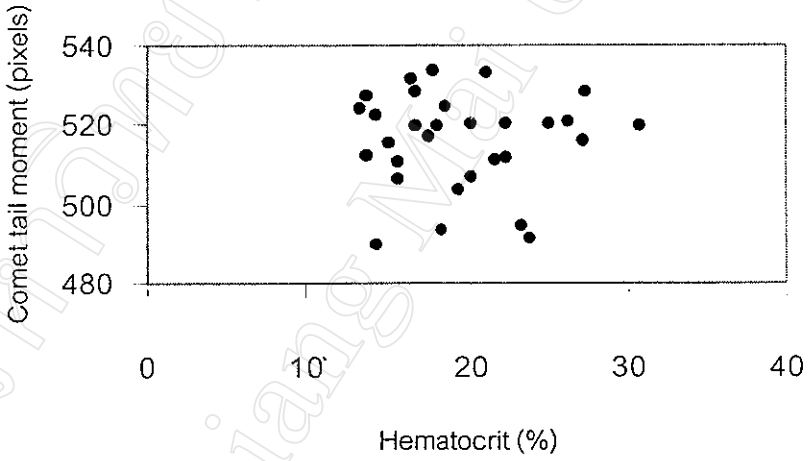

Figure 14. Correlation of hematocrit and comet tail moment of NRBC β-thalassemic patients (n=30).

3. **Correlation of red blood cell count and comet tail moment of NRBC β-thalassemic patients.**

Red blood cell count and comet tail moment of NRBC β-thalassemic patients (mean ± SD) were 2.56 ± 0.65 x 10^6 cells/μL and 515.70 ± 12.10 pixels respectively ($r = -0.07$, $p = 0.05$) as shown in Table 11. A decreased red blood cell count was also observed in thalassemic patients compared with normal cord blood (2.56 ± 0.65 *versus* 4.11 ± 0.49 x 10^6 cells/μL, $p < 0.05$). The high comet tail moment of NRBC showed a lot of DNA strand break.

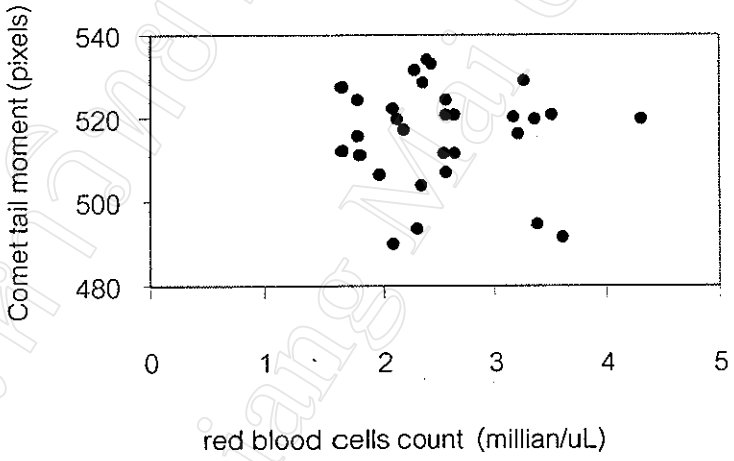

Figure 15. Correlation of red blood cell count and comet tail moment of NRBC β thalassemic patients (n=30).

4. **Comparison comet tail moment of NRBC between normal cord blood and β-thalassemic patients**

Mean ± SD of comet tail moment were 88 ± 2.5 pixels in normal cord blood (n=5) and 515.17 ± 12.10 pixels in β-thalassemic patients (n=30) which were higher significant by $p<0.05$. This revealed that there is a little DNA damage in NRBC normal cord blood while a lot of DNA strands break in β-thalassemic patients as shown in Figure 12.

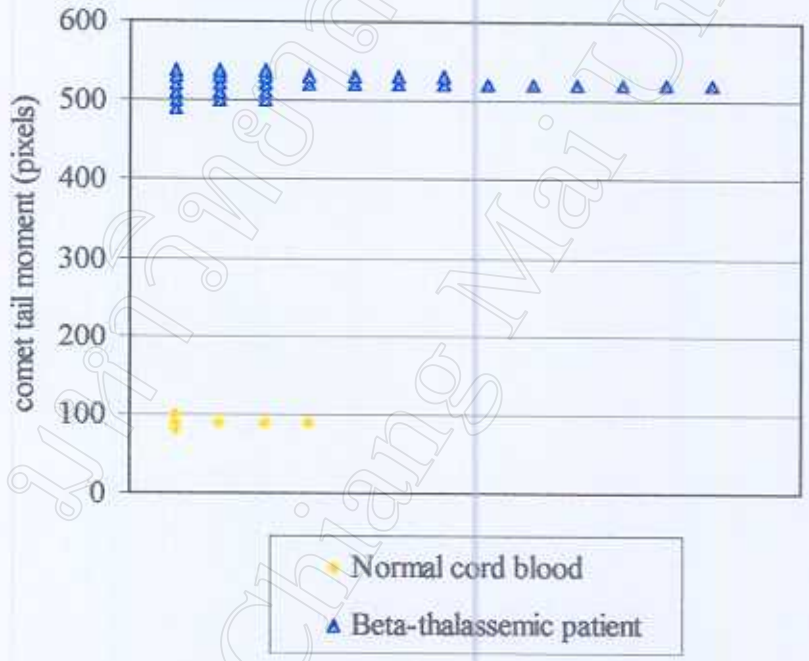

Figure 16. Comparison comet tail moment of NRBC between normal cord blood and β-thalassemic patients.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

5. Comparison comet tail moment of PBMC between normal cord blood and β-thalassemic patients

Mean ± SD of comet tail moment in PBMC of normal cord blood and β-thalassemic patients (n=30) were 28 ± 2.5 pixels and 165.17 ± 2.10 pixels, respectively. The comet tail moment of PBMC of β-thalassemic patients were significantly higher than normal cord blood ($p<0.05$).

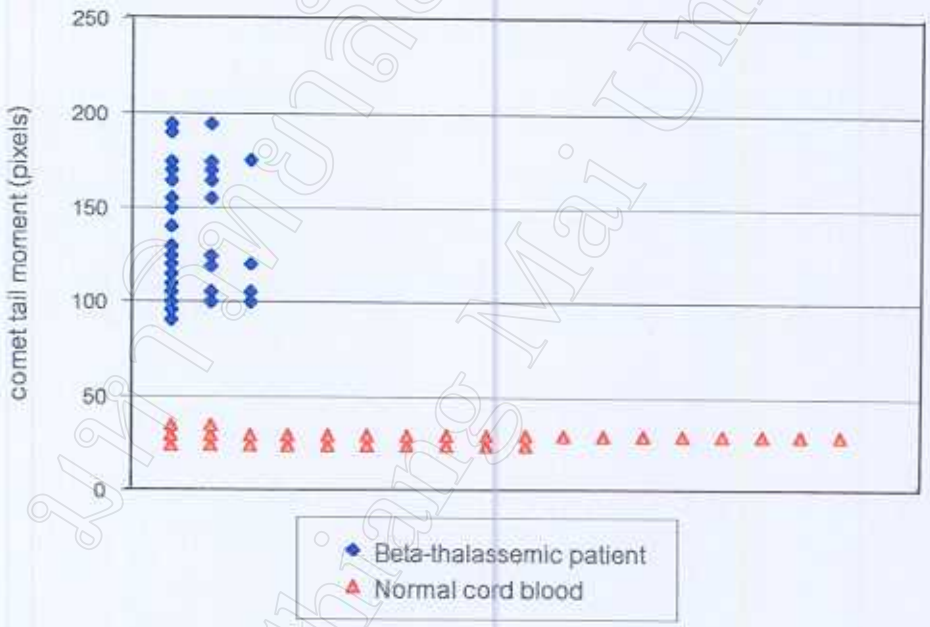

Figure 17. Comparison comet tail moment of PBMC between normal cord blood and β-thalassemic patients.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

PART III

1. **The effect of β-thalassemic plasma on cells oxidative stressed and apoptosis.**

An important consideration was β-thalassemic plasma could induce cell oxidatively stressed and apoptosis. This study had done in *vitro* and the method was modified form Banjadpongchai R, *et al.*, 1997. PBMC from normal cord blood were analyzed by treating each cell with β-thalassemic plasma (v/v).

The results showed that when β-thalassemic plasma was added to the supplement media instead of fetal calf serum at concentration 10, 20, 30, 40 and 50 % for 12 hours, the viability of PBMC were 84, 71, 56, 33 and 19 % respectively. After these cells were analyzed by comet assay, it was found that β-thalassemic plasma which added to the supplement media could induce DNA damage. An increased of comet tail moment was also observed in treated PBMC cord blood compared with untreated cells (164 ± 27.60, 300 ± 44.14, 434 ± 51.69, 499 ± 46.17 and 535 ± 12.08 versus 26 ± 2.89 pixels, $p< 0.05$).

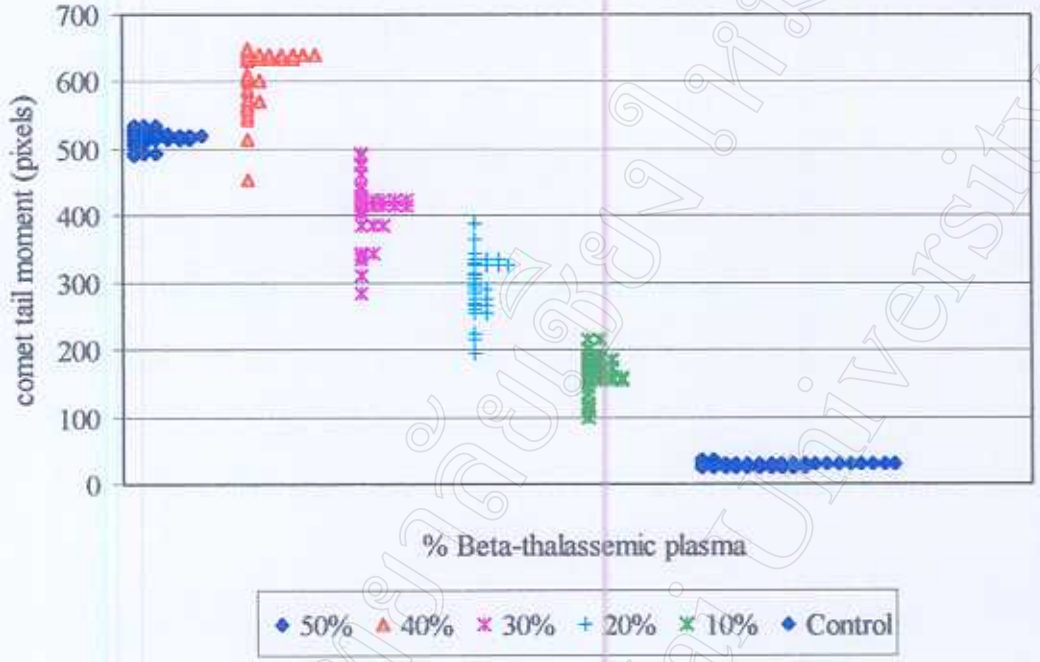

Figure 18. Effect of β-thalassemic plasma on cell oxidatively stressed and apoptosis.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

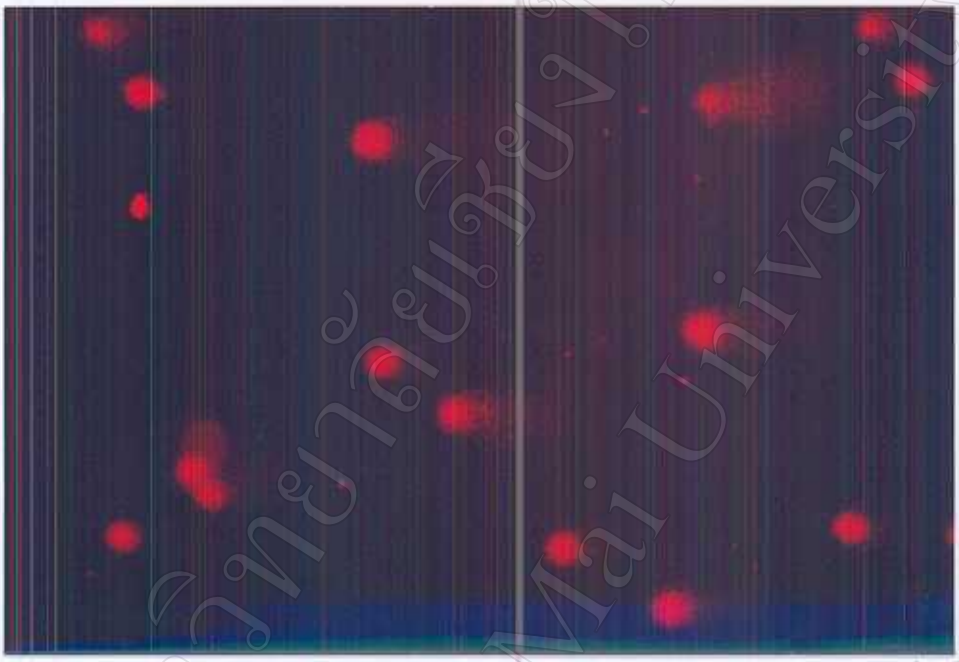

Figure 19. Comet cells treated with 10% β-thalassemic plasma: 160X

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

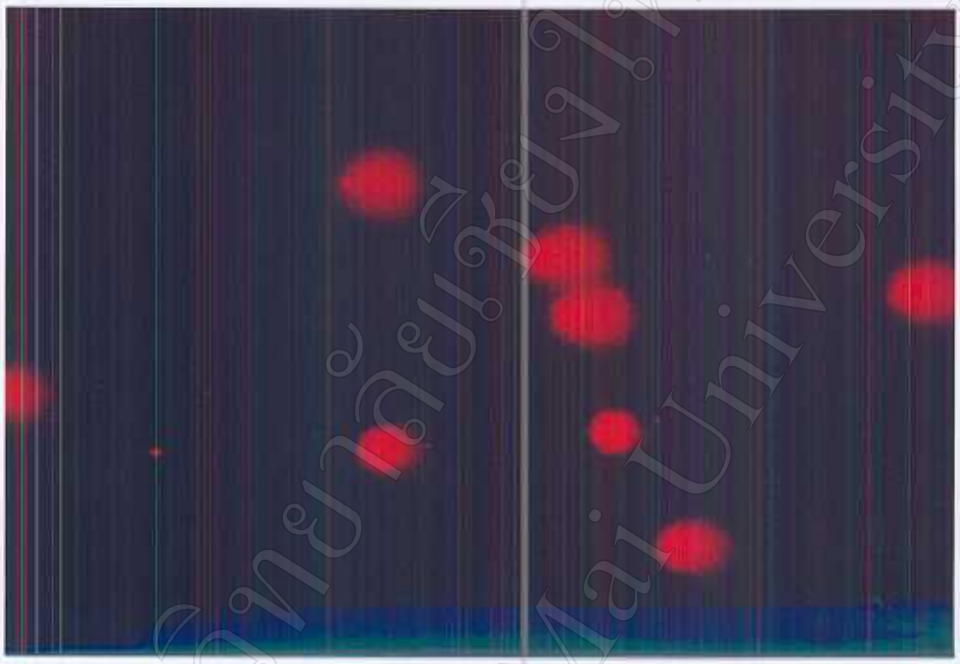

Figure 20. Comet cells treated with 20% β-thalassemic plasma: 160X

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

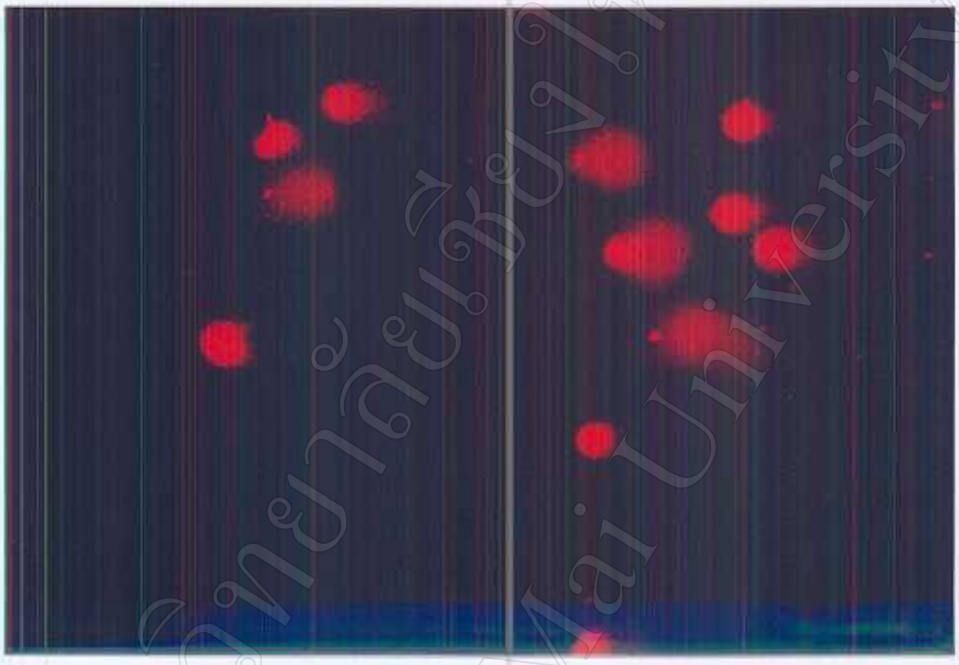

Figure 21. Comet cells treated with 30% β-thalassemic plasma: 160X

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

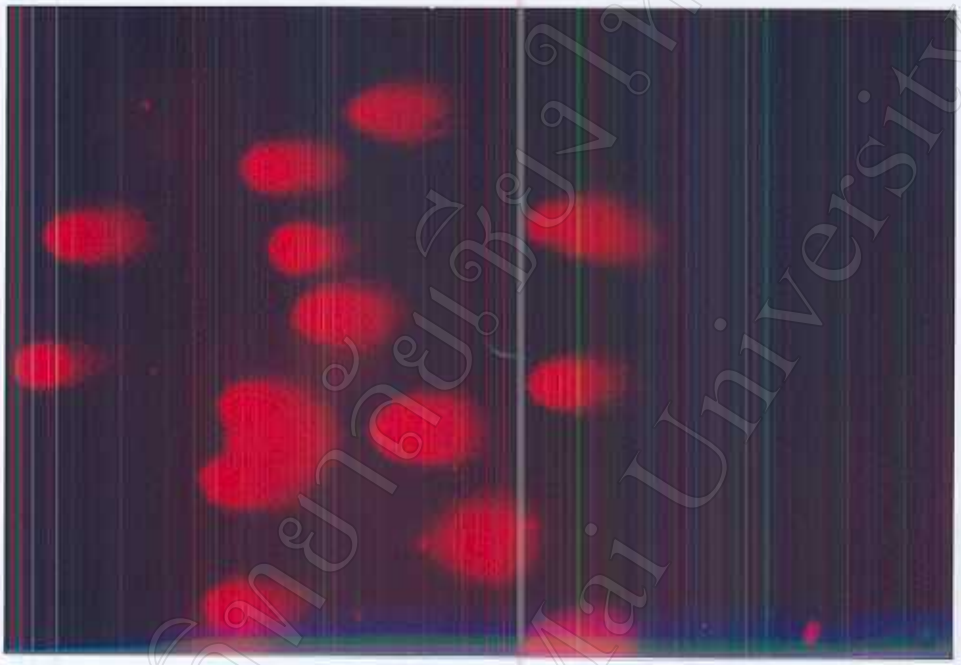

Figure 22. Comet cells treated with 40% β-thalassemic plasma: 160X

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

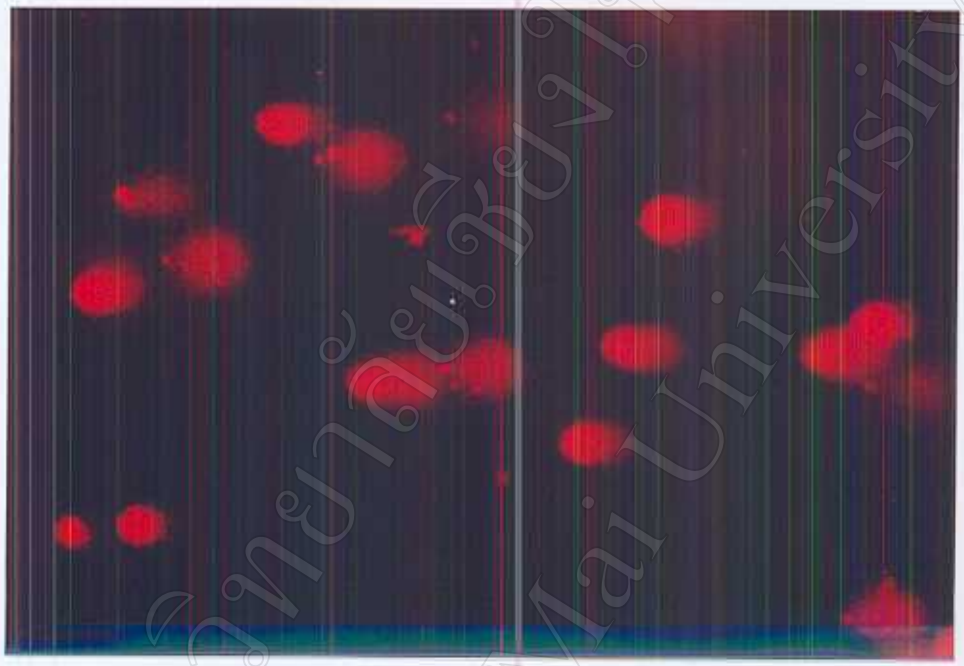

Figure 23. Comet cells treated with 50% β-thalassemic plasma: 160X

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

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

An important consideration was whether iron and H_2O_2 could induce cell oxidatively stressed and apoptosis. This study was done in the *vitro* and the Fenton reaction was modified from Suttajit M, *et al.*, 2000.

2.1 **Titration of Fenton reaction**

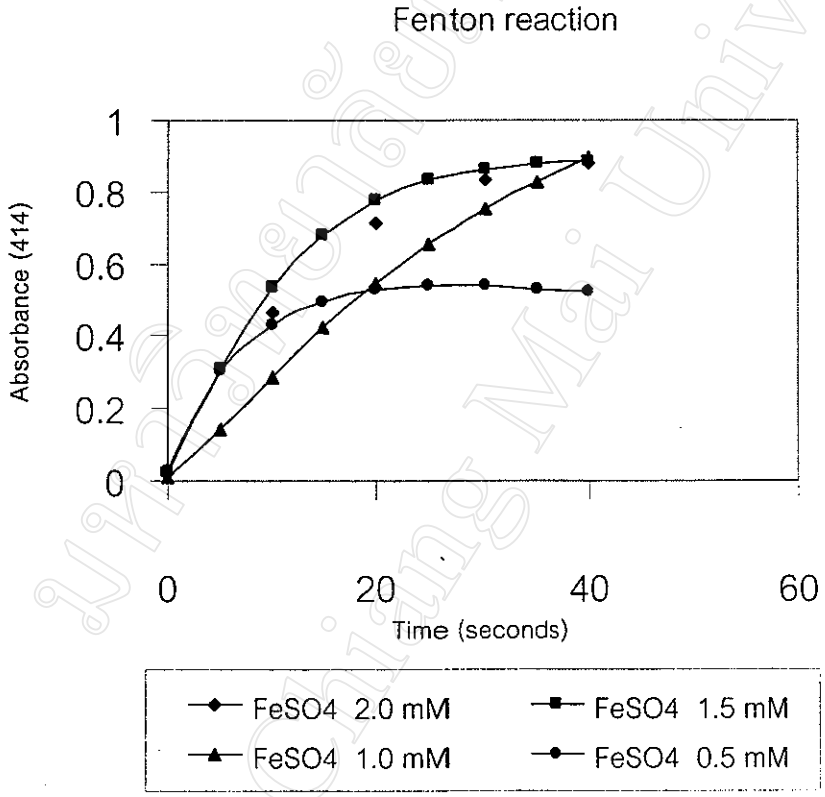

Figure 24. Fenton reaction: the optimum concentration which had linearity curve been 1.0 mM $FeSO_4$ and 500 μM H_2O_2.

2.2 **Cytotoxicity test (Trypan blue exclusion)**

Determination of cytotoxicity of $FeSO_4$ and H_2O_2 to normal cord blood PBMC. Table 9 and 10 showed the cell viability up to 90% were showed at 0.5 mM $FeSO_4$ and 50 μM H_2O_2..

Table 8. Viability of cell when treated with $FeSO_4$.

Concentration (mM)	% viability
0.25	96
0.5	91
1.0	87
2.5	53
5.0	31

Table 9. Viability of cell when treated with H_2O_2.

Concentration (μM)	% viability
25	98
50	93
100	83
250	76
500	49

2.3 **Effect of iron ($FeSO_4$) and H_2O_2 on cell oxidatively stressed**

The effects of iron ($FeSO_4$) on cells oxidatively stressed with hydrogen peroxide (H_2O_2) were done *in vitro*. Both human PBMC from normal cord blood and β-thalassemic patient showed an increase in DNA damage measured by the comet assay after treatment with Fenton reaction. The comet tail moment (mean ± SD) of untreated cells (PBMC normal cord blood and β-thalassemic patients) were 25.89 ± 2.99 pixels and 136.20 ± 28.58 pixels respectively. While $FeSO_4$ and H_2O_2 treated cell, the comet tails moment were 459.19 ± 48.73 and 590.59 ± 62.83 pixels respectively. An increased of comet tail moment were also observed in $FeSO_4$ and H_2O_2 treated cells compared with untreated cells ($p< 0.05$).

We observed that the Fenton reaction induced DNA damage in a dose-related response. PBMC from β-thalassemic patients had a comet tail moment higher than normal cord blood when used equal dose of $FeSO_4$ and H_2O_2.

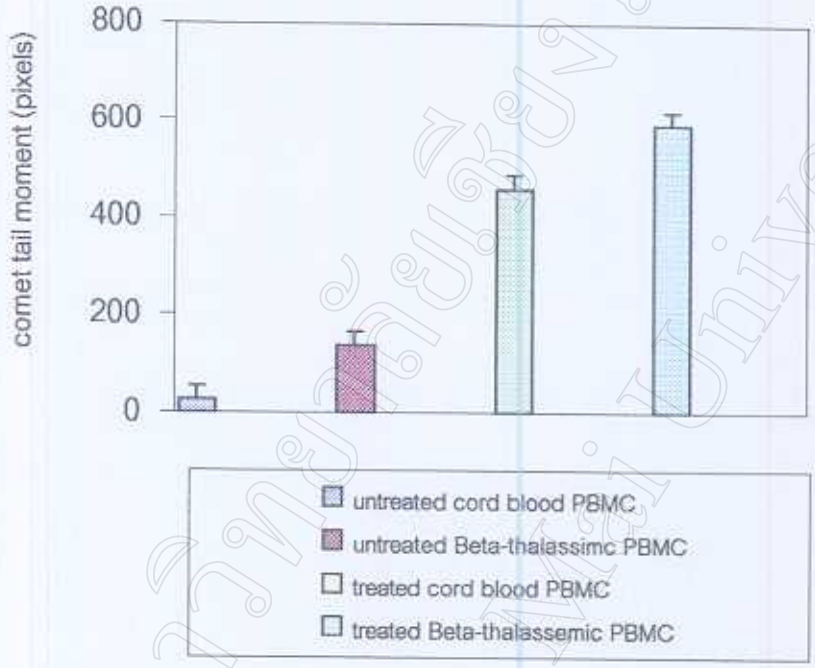

Figure 25. Iron ($FeSO_4$) and H_2O_2 on cell oxidatively stressed. The cells were treated with 1.0 mM $FeSO_4$ and 50 μM H_2O_2 in modified G-buffer pH 7.2 for 4 hours at 37 °C in 5 % CO_2 and effect of oxidative stress was measured by comet assay.

มหาวิทยาลัยเชียงใหม่ Chiang Mai University

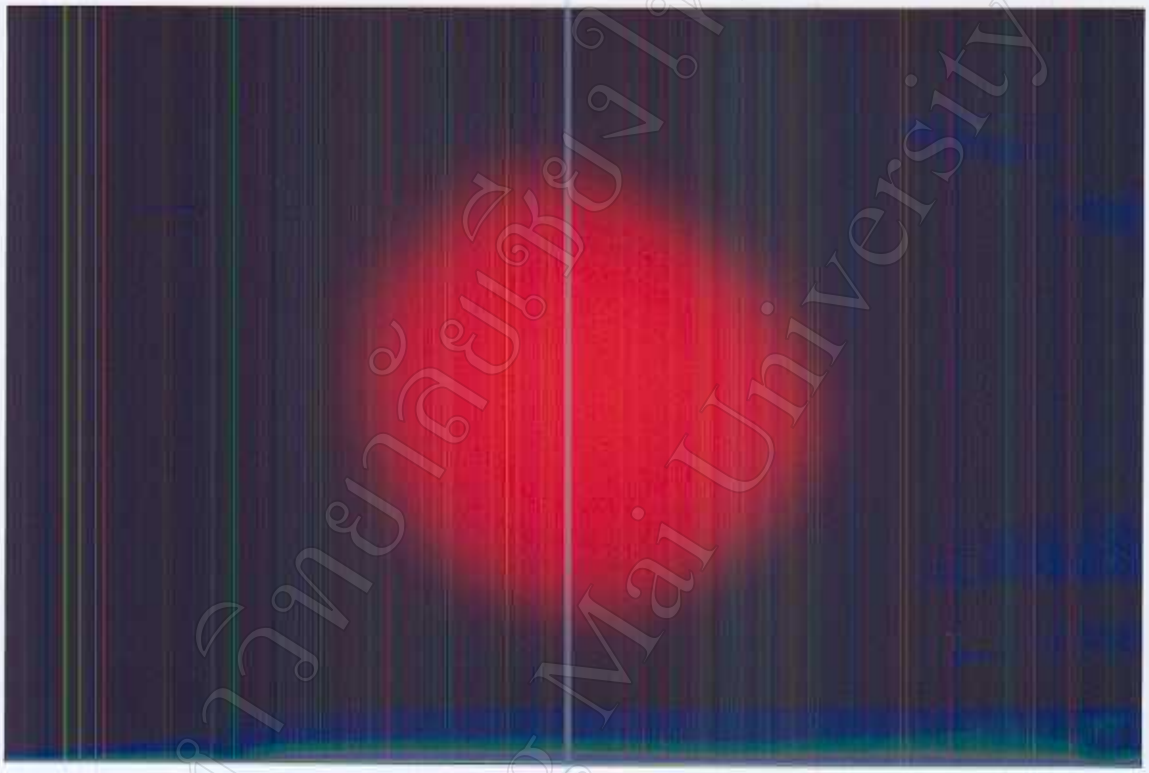

Figure 26. Comet cell with untreated $FeSO_4$ and H_2O_2: 1000X

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

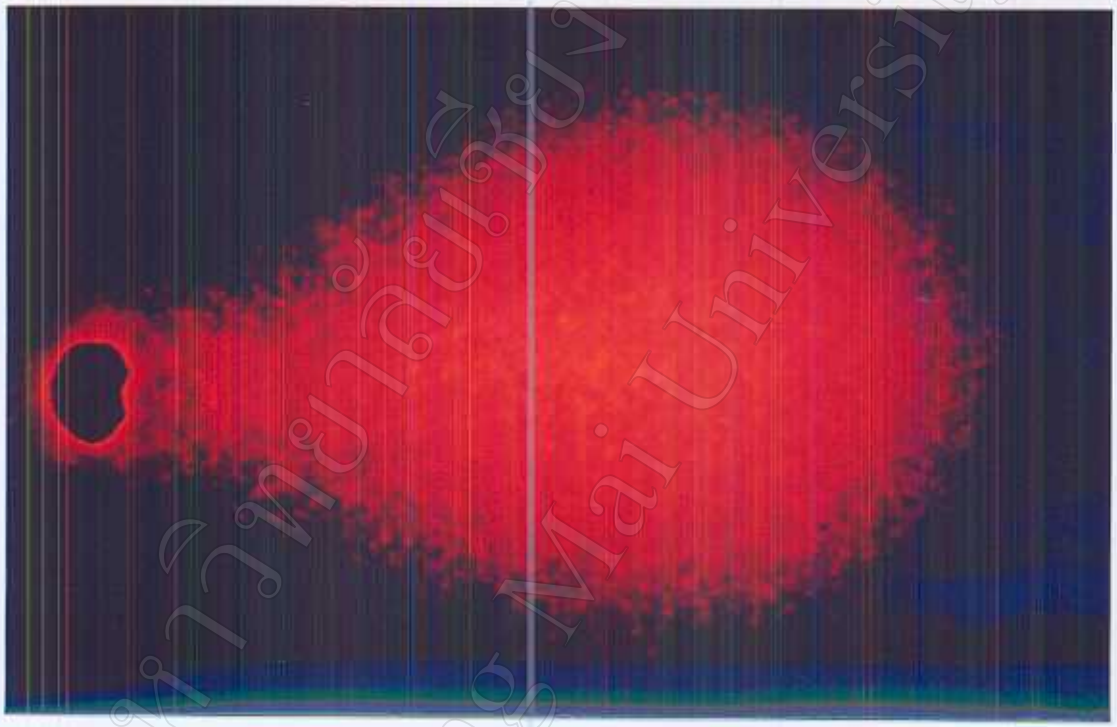

Figure 27. Comet cell with treated $FeSO_4$ and H_2O_2:1000X

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

3. **The effect of curcumin to inhibit oxidative stress and apoptosis**

3.1 **Test thin layer chromatography (TLC)**

Curcumin content in turmeric is about 1-5 %, which has been identified as a major yellow pigment isolated from the ground rhizome of the *Curcumon species.* Three major curcumin namely curcumin, demethoxycurcumin and bisdemethoxy curcumin were extracted by absolute ethanol in a mixture curcuminoid. The curcuminoid were tested thin layer chromatography (TLC) compare with commercial curcumin (79:19:2) ICN Bio-medical (Hyland Ave., CA, USA).

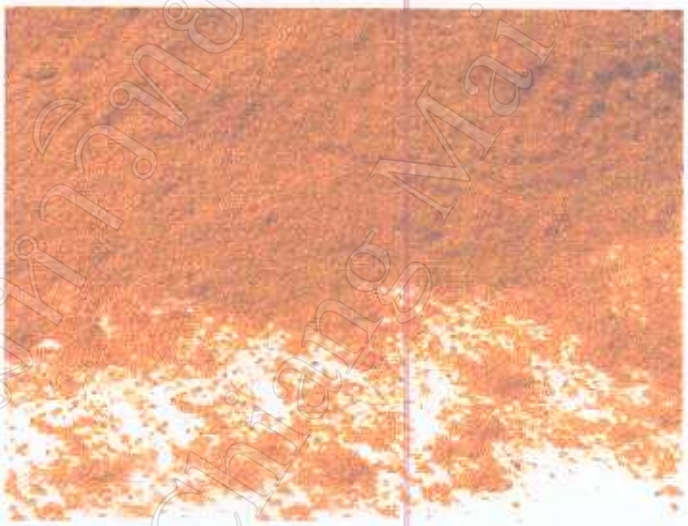

Figure 28. Turmeric powder.

Figure 29. Separation of curcumin, demethoxycurcumin and bisdemethoxy curcumin by TLC. There was no difference in region of band between the commercial curcumin and turmeric extract. The rate of flow (RF) of three bands was 0.34, 0.43 and 0.53 both of curcuminoid.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

3.2 **Cytotoxicity test (Trypan blue exclusion)**

Determination cytotoxic of curcumin to cord blood PBMC was modified from Limtrakul P, *et al.*, (1997).

Table 10. Viability of normal cord blood PBMC when treated with curcuminoid.

Concentration (μg/mL)	% viability
1	98
5	94
10	90
15	88
20	73
25	51

Cell viability of turmeric extract treated normal PBMC cord blood for 12 hours. The cell viability up to 90% were showed at concentration between 1-10 μ g/mL turmric extract treated PBMC.

3.3 **Test % inhibition of oxidation of commercial curcumin.**

In this study we measured the activity of curcumin on the inhibition of oxidation reaction.

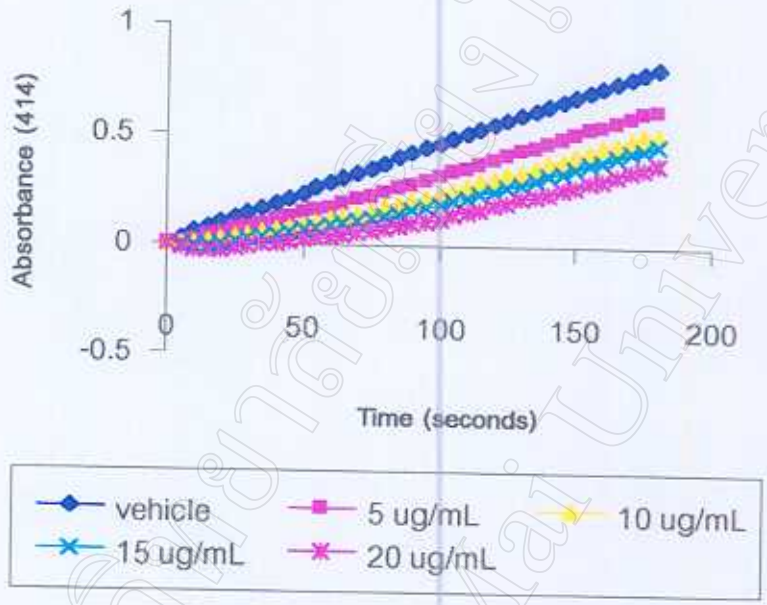

Figure 30. The inhibition of oxidation reaction by commercial curcumin. The result showed that commercial curcumin at concentrations of 5, 10, 15 and 20 µg/mL demonstrated a dose dependent manner inhibition of the oxidation reaction by methmyoglobin-ABTS method.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

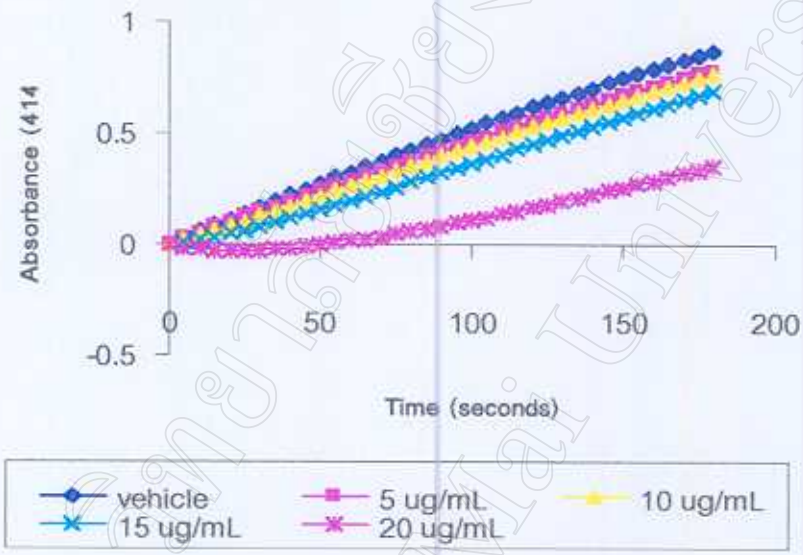

Figure 31. The inhibition of oxidation reaction by turmeric extract. The result showed that turmeric extract at concentrations of 5, 10, 15 and 20 μg/mL demonstrated a dose dependent manner inhibition of the oxidation reaction by methmyoglobin-ABTS method.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

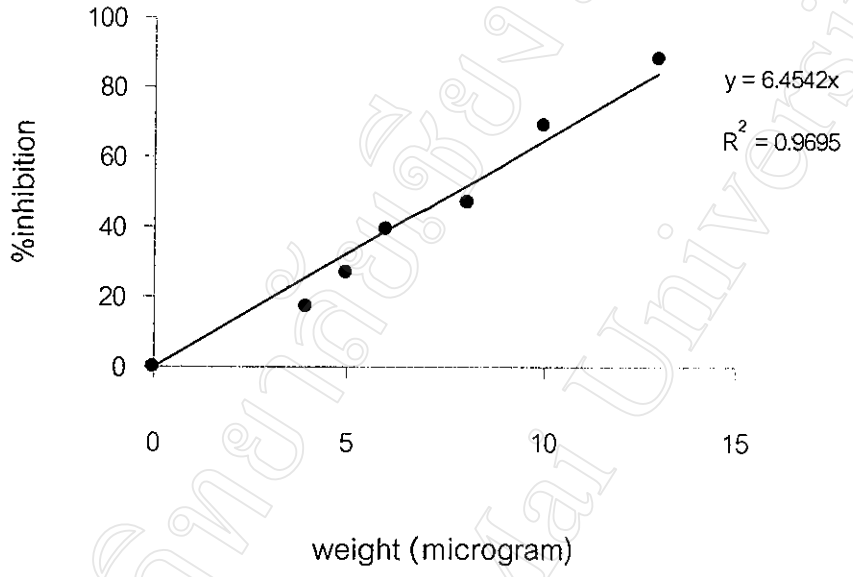

Figure 32. %Inhibition oxidation of Trolox. The result showed % inhibition oxidation of Trolox. There was a dose dependent manner of Trolox to inhibit the oxidation reaction when used at concentration 4, 5, 6, 8, 10 and 13 μg/mL respectively. In this study we observed that the Trolox equivalent antioxidative capacity (TEAC) was 6903/ 1 mg.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

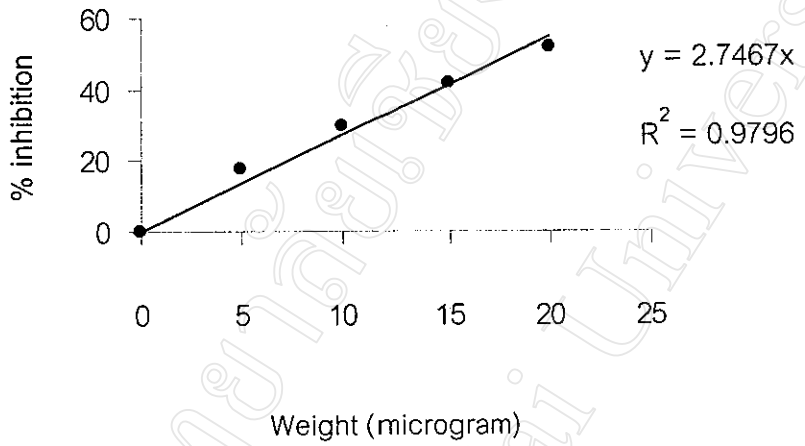

Figure 33. % Inhibition oxidation of commercial curcumin. There was a dose dependent of commercial curcumin (curcumin, demethoxycurcumin and bisdemethoxycurcumin, 79:19:2 %) to inhibit the oxidation reaction when used at concentration 5, 10, 15 and 20 μg/mL respectively. In this study we observed that antioxidative capacity of commercial curcumin was 3000/1 mg and Trolox equivalent antioxidative capacity (TEAC) was 0.4345.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

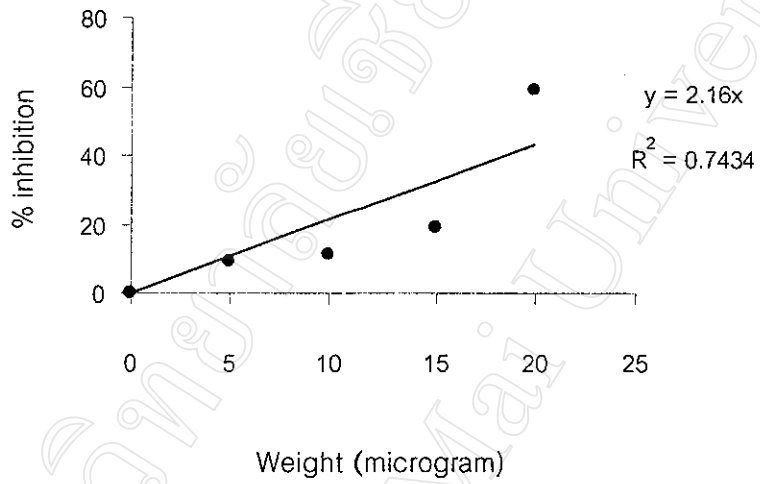

Figure 34. % Inhibition oxidation of turmeric extract. There was a dose dependent manner of turmeric extract to inhibit the oxidation reaction when used at concentration 5, 10, 15 and 20 μg/mL respectively. In this study we observed that the antioxidative capacity of extracted curcumin was 1100/ 1 mg and Trolox equivalent antioxidative capacity (TEAC) was 0.1593.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

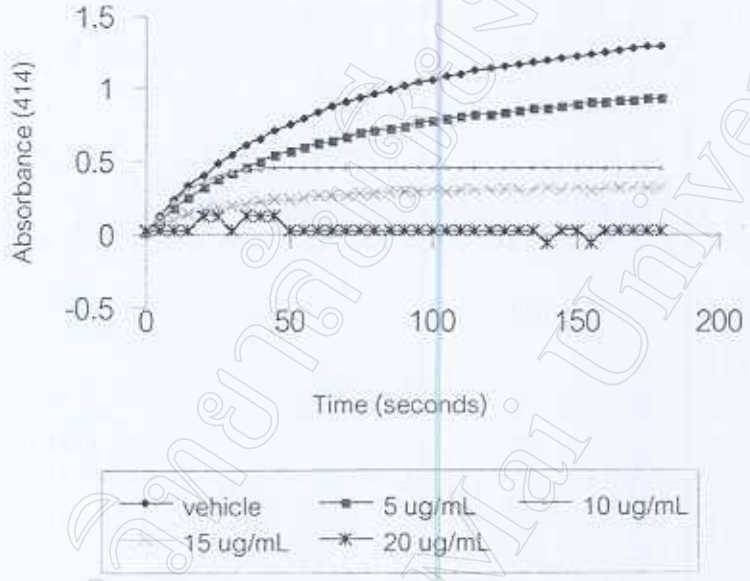

Figure 35. Inhibition oxidation of Fenton reaction by turmeric extract. There was a dose dependent manner of turmeric extract that could inhibit Fenton reaction when used at 5, 10, 15 and 20 μg/mL respectively. Activity of curcumin that could inhibit Fenton reaction.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Chiang Mai University

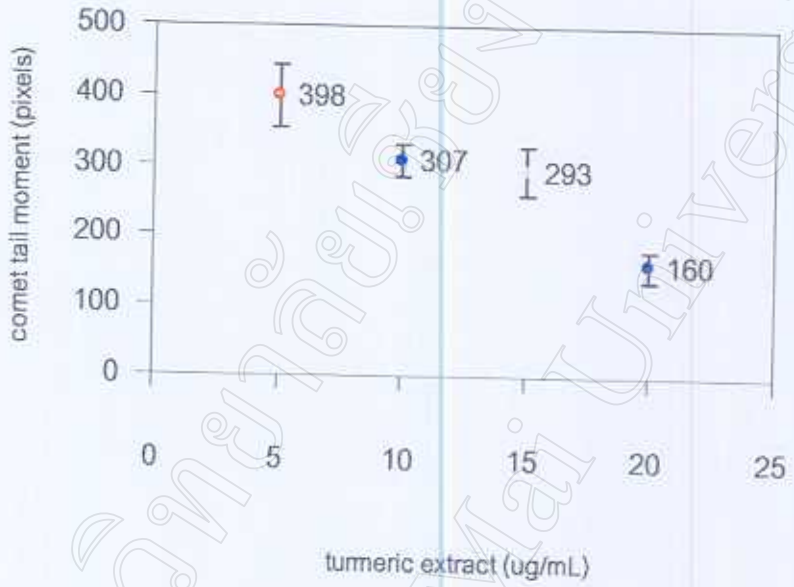

Figure 36. Effect of turmeric extract to inhibit oxidative stress on cells. When cotreated PBMCs with turmeric extract at concentration 5, 10,15 and 20 μg/mL in Fenton reaction ($FeSO_4$ and H_2O_2). The comet tail moment were decreased when added an increase concentration of turmeric extract. There was a dose-related response to inhibit oxidative stress.

มหาวิทยาลัยเชียงใหม่ Chiang Mai University

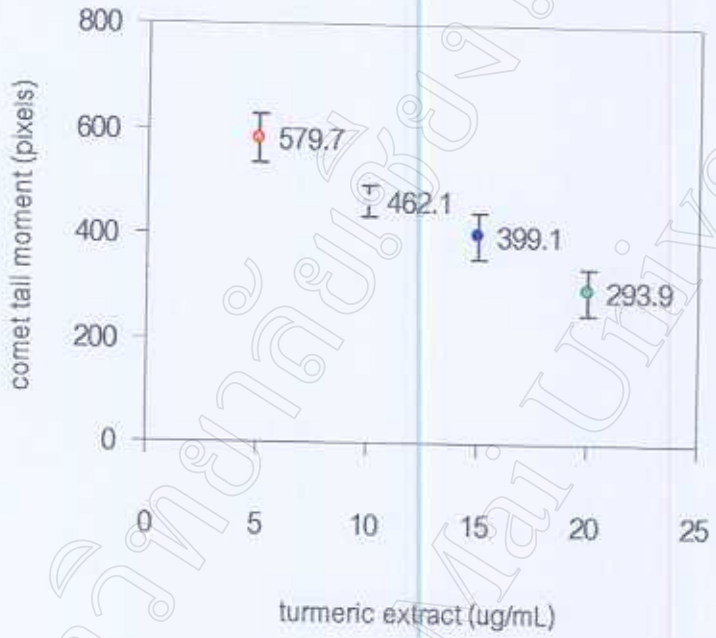

Figure 37. Effect of turmeric extract to inhibit oxidative stress on cells. When pretreated PBMCs with turmeric extract at concentration 5, 10, 15 and 20 μg/mL at 37°C in 5 % CO_2 for 1 hour and then treated cells with Fenton reaction. There was a dose-related response to inhibit oxidative stress.

มหาวิทยาลัยเชียงใหม่ Chiang Mai University