IV. DISCUSSION

Apoptosis or programmed cell death was a mode of cell death occurred in physiological and pathological conditions e.g. AIDS, autoimmune disease, neurodegenerative disorders. This kind of death was characterized using two or three criteria such as morphology or biochemical or cell biological basis. The typical morphology of apoptotic cells includes condensed nuclei, compact cytosol, cell membrane blebbing, and fragmented bodies. The DNA character or biochemical criterion was ladder pattern on agarose gel electrophoresis in the late stage due to the internucleosomal cleavage.

Rydberg B and Johnsonln KJ 1978 introduced comet assay or single cell gel electrophoresis as a method to measure DNA damage in individual cells. In the years following, a great understanding of the biological details of apoptosis along with advancements in digital imaging have helped establish the comet assay as one of the suitable methods to measure cellular DNA damage. The use of the comet assay has grown considerably for research applications in many scientific disciplines. The assay was named for the characteristic shapes seen when the DNA exited the nucleus and cells body, and migrated toward the anode.

The comet assay worked because DNA strand breaks created fragments that, when embedded in an agarose gel, migrated in an electric field. The strand breaks damage the higher order, tightly packed structure of DNA, which also allowed migration outside the region of the cell nucleus. Different assay conditions allowed the study of either single-strand or double-strand DNA damage. Alkaline treatment

facilitated the unwinding and denaturation of the DNA molecules, allowing for the sensitive detection of single-strand damage. Neutral assay conditions, on the other hand, do not induce denaturation and consequently allow the detection of only double-strand damage. The ability to analyze these two endpoints separately was another advantage of the comet assay.

Slides for the comet assay were prepared by mixing a suspension of cells with liquid agarose, spreading out the suspension in a thin layer on a microscope slide and allowing the mixture to solidify. Cells were lysed in alkaline condition and DNA-bound proteins were removed so as not to interfere with migration in an electric field. Sufficient washing of the gels in solutions of the appropriate pH help to remove all other ions that might alter the electrophoretic conditions. Electrophoresis at low voltage then allowed the migration to occur in a controlled manner, after which the cells were stained with a DNA-binding fluorescent dye for visualization and image analysis of the "comet".

The best way to quantify the amount of DNA damage using a digital comet image was combing tail length and the distribution of DNA in the tail. These two important comet quantities define the "tail moment". At first, many thought that tail length alone might be a sufficient indicator of DNA damage, as higher degree of damages would result in more and smaller fragments which would result in longer tails. However, it had since been widely illustrated that tail length alone was not the best factor because tail length would increase for low levels of damage.

Olive PL, *et al.*, (1990) introduced the tail moment approach, which combined these two factor. The tail moment was calculated as a true physical tail moment about the center of comet head. Intensity integrals for each column of pixels data were

weighted by multiplying by their horizontal distance (in pixels) from the center of the comet head. These individual "moment" were then summed for the entire comet tail and normalized to the total comet intensity to give the tail moment.

Simply put, if the DNA intensities in pixels values represent mass, the tail moment was the distance from the center of comet head to the tail's center of mass, multiplied by the fraction of the DNA in the tail.

Massive fragmentations of cellular DNA made apoptotic cells easy to distinguish using the comet assay, as almost the entire volume of DNA migrated outside the head of the comet. Figure 38 illustrated this point, as most of DNA of healthy cells remained in the cell nucleus with very little migration in the electric field outside of the cells. The DNA of apoptotic cells exited the cell body and migrated through the gel forming pronounced comet tail (Figure 39). The stark contrast in shapes between these images might allow the determination of apoptotic cell fractions based solely on empirical scoring.

The value for calculated tail moment for apoptotic cells were extremely high due to the large fraction of DNA in the tail while undamaged cells retain low tail moments. In general, the magnitude of the tail moment values would differ for different systems due to differences in magnification and digitization. Using this Kinetic Imaging, Zeiss Microscope model axioskop 2 both fluorescent and light function linked to a CCD camera of NRBC or PBMC, a tail moment of more than 400 pixels was indicative of apoptosis.

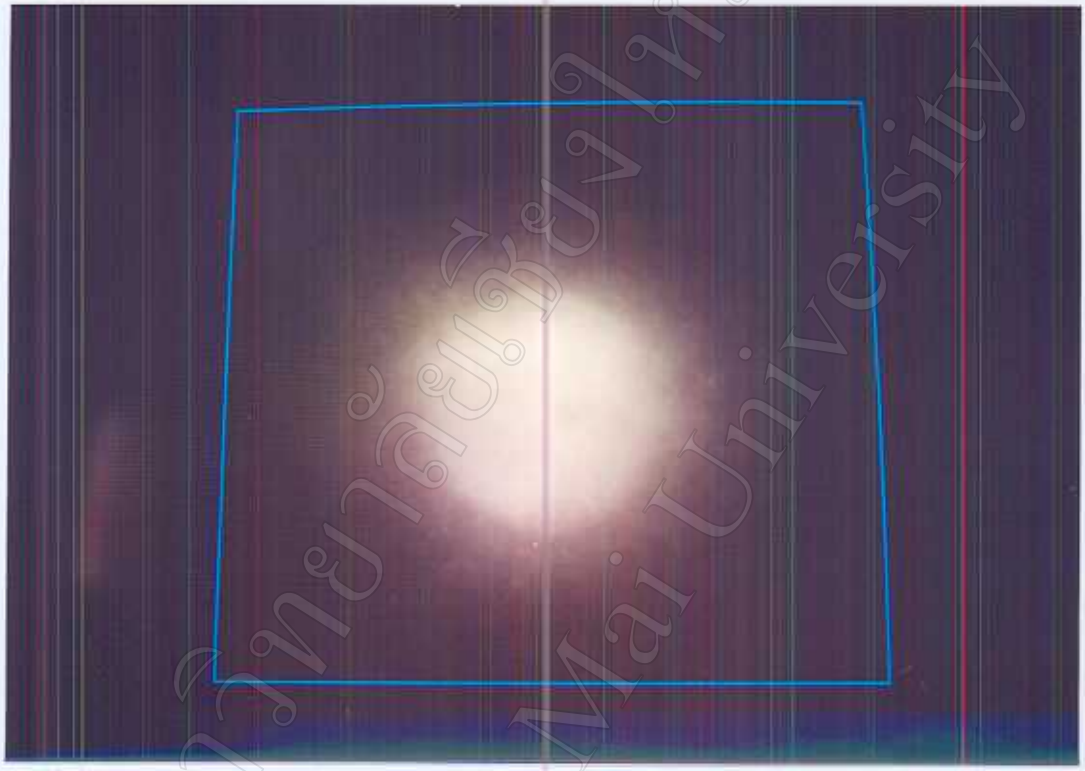

Figure 38. Digital image of PBMC embedded in agarose, subjected to the comet assay. Control cell with an undetectable degree of DNA fragmentation, resulting in the cellular DNA remaining in the nucleus.

Figure 39. Digital image of PBMC embedded in agarose, subjected to the comet assay. Apoptotic cell showing severe fragmentation of cellular DNA, evidenced by a mass migration of the DNA out of the cell body (comet head) and into the surrounding agarose (comet tail).

In this study the hematological indices of 30 β-thalassemic patients showed severe anemia when compared with normal cord blood. This result contributed ineffective erythropoiesis in the bone marrow and peripheral hemolysis of red blood celsl. Peripheral hemolysis of RBCs might contribute to the anemia more in the severe α-thalassemia (HB H disease) but intramedullary hemolysis was the major kinetic defect that lead to anemia in β-thalassemia major (Finch CA, *et al.*, 1970).

The role of programmed cell death (apoptosis) had been shown for many tissue and cell lines (Koury MJ and Bondurant MC, 1990). Apoptosis was a selective process of physiologic cell deletion and plays a major role in the control of normal and abnormal processes (Raff MC, 1992). Apoptosis has been described in erythroid precursors *in vitro* system and found that no evidence for apoptosis in normal erythropoiesis (Koury MJ and Bondurant MC, 1990).

In the previous studied Yuan J, *et al.*, (1993) showed that the accelerated apoptosis in erythroid precursors of patients with severe β-thalassemia (Cooley's anemia). There was obvious DNA ladder product in β-thalassemia major, with less occurring in β-thalassemia trait. Normal individuals showed only a slight smear of breakdown of DNA. These results indicated there is enhanced apoptosis in the erythroblasts in the bone marrows of β-thalassemic patients.

The kinetics of sequential erythroid differentiation in β-thalassemia major was studied by Mathias LA, *et al.*, (2000), they found that erythroid progenitor cells of β-thalassemia expanded 10 to 20 fold less than normal bone marrows in erythroid culture conditions. There were less viable cells during differentiation, specifically after the polychromatophilic normoblast. There was a progressive increase in the

apoptotic erythroid progeny with differentiation, and apoptosis occurred predominantly at the polychromatophilic normoblast in β-thalassemia major.

In present study we evaluated the possibility that severe anemia in β-thalassemic patients might be cause of apoptosis in erythroid cells. Hematological indices (hemoglobin, hematocrit and red blood cell count) were used to show anemia in patients and comet assay used to measure apoptosis in NRBC and PBMC (comet tail moment was used to demonstrated a lot of DNA strand break of apoptotic cells).

In this study the patients who post splenectomy were selected. An decreased of hemoglobin, hematocrit and red blood cell count were also observed in patients compared with normal cord blood. An important consideration was whether severe anemia was characterized by increased NRBCs apoptosis. The result showed severe anemia and apoptosis in NRBCs of β-thalassemic patients. Thalassemic patients had a preponderance of high value in comet tail moment (5 times more than normal cord blood), the high value that suggested a lot of DNA strand break occurred in NRBCs of β-thalassemic patients. Consideration was whether severe anemia was characterized by increased NRBCs apoptosis the result showed a least correlation (in negative trend) with comet tail moment of NRBC β-thalassemic patients. Variability of hemoglobin, hematocrit and red blood cell counts were found in these patients. This evidence suggested these patients had a different level of anemia (ex. mild, moderate and severe anemia). The type of β-thalassemia, hemoglobin concentration, iron over load, low level of anti-oxidant and chronically transfusion-dependent might be cause of variability anemia in patients.

A high value of comet tail moment in NRBCs of β-thalassemic patients might be showed a decreased of the hematological indices (hemoglobin, hematocrit and red blood cell count). When NRBCs and PBMCs of normal cord blood and thalassemic patients were measured apoptosis the result showed a little DNA damage in normal cord blood while undamaged DNA was found in PBMC of cord blood (comet tail moment: 88 ± 2.5 and 25 ± 2.5 pixels respectively). The comet tail moment of NRBCs normal cord blood was slightly greater than PBMCs, this evidence suggested the nucleus of normal cord blood had nuclear alterations.

Shinar E and Rachmilewitz EA, 1990 have shown oxidative denaturation of red blood cells in thalassemia excess of iron was a catalyst of peroxidation *via* the Fenton reaction, causing damage to the various RBC membrane components (lipids. proteins, etc).

Factor affecting induced apoptosis were elucidated in this study, PBMC from normal cord blood and β-thalassemic patients was determinated. The effect of β-thalassemic plasma on cell oxidatively stressed was modified from Banjerdpongchai R, *et al.* (1997). PBMC from normal cord blood and patient were analyzed by treating the cells with β-thalassemic plasma (v/v). The result was found that thalassemic plasma had a cause and effect as a dose response relationship to cause DNA damage of both PBMC (normal cord blood PBMC and β-thalassemic patients) to percentage of thalassemic plasma, i.e. increased percentage of thalassemic plasma would increased in DNA damage of PBMC. This result might be explained that plasma from thalassemic patient had an increased oxidant which produced by several evidences for instance, low level of vitamin E and high level of lipid peroxide

(Banjerdpongchai R, *et al.*, 1997). Then, the oxidants damaged PBMC and lead to apoptosis *in vitro*.

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-transferrin iron. The leakage of the red cell contents such as Fe^{2+} and hemin had been found to induce the oxidative damage or trigger ROS production (Anderson D, *et al.*, 2000)

The role of Fe^{2+} on oxidant injury to the PBMC was intriguing since Fe^{2+} was a catalyst capable of simulating reactive oxygen species (ROS) generation, e.g. hydroxyl radical ($OH^{\bullet}$). Hydrogen peroxide could readily react with transition-metal catalysts to $OH^{\bullet}$.

Hydroxyl radicals ($OH^{\bullet}$) were the most reactive oxygen free radical species capable of direct oxidative damage to macromolecules including DNA, protein, and lipid membrane. Effect of excess results of oxygen free radicals, such as DNA strand breaks and membrane blebbing, match the hallmark feature of apoptosis. The effect of iron salt on cells oxidatively stressed measured in the comet assay was studied by Anderson D, *et al.*, 2000. They found that the human lymphocytes from a male and a female donor and human adenocarcinoma colonic cells showed an increased in DNA damage in comet assay after treatment with H_2O_2. Ferric chloride produced an increased in DNA damage in human adenocarcinoma colonic cells, but little or no damaged in human lymphocytes. Ferrous chloride also produced weak DNA damaged in human lymphocytes, but ferrous sulphate produced a dose-related response.

The effect of iron overload and H_2O_2 on cells oxidatively stressed and apoptosis was elucidated. In this study, 1.0 mM $FeSO_4$ and 50 μM H_2O_2 were induced oxidative stress to both PBMC (normal cord blood and β-thalassemic patient), the

result was cell death *via* apoptosis. It was found that PBMC of patients had an increased DNA damage compared with normal cord blood when treated cells with equal dose of $FeSO_4$ and H_2O_2, this evidence demonstrated PBMC of patients might be more sensitive to stimulated iron overload with H_2O_2.

Fe^{2+} and Cu^{2+} were oxidized transition metal ion that readily accessible reduced states and, furthermore, were present in high enough abundance in many tissue to make them reactants for one-electron oxidation or reductions that could generate reactive free radicals. Agents that could bind reactive transition metal cations by complexation could decrease their biological effect. In nature, there were many agents which probably could chelate metal ions strongly enougt to make them effective antioxidants. Flavonoids had also been demonstrated to form stable complexes with Cu^{2+} cations (Thompson M, *et al.*, 1976).

Curcumin has a wide range of biological and pharmacological activities including hypocholesterolemic effects in rats (Rao DS, *et al.*, 1970), hypoglycemic effects in man (Srinivasan M, *et al.*, 1972) and antithrombotic (Srivastava R, *et al.*, 1985).

Curcumin is a potent scavenger of a variety of ROS, including superoxide anion (Kunchady E and Rao MNA, 1990), hydroxyl radical, singlet oxygen (Subramanian M, *et al.*, 1994), nitric oxide and peroxynitrite. Curcumin has the ability to protect lipids, hemoglobin, and DNA against oxidative degradation. Pure curcumin has more potent superoxide anion scavenging activity than demethoxycurcumin or bisdemethoxycurcumin (Kunchady E and Rao MNA, 1990). Curcumin is also a potent inhibitor of ROS-generating enzyme cycloxygenase and lipoxygenase in mouse epidermis (Huang MT, *et al.*, 1991).

The comet assay has been explored as potential tool for detecting the antioxidant effect of foods or nutrients. Supplementation with curcumin at 1, 5, 10, 15, 20, 25 μg/mL for 12 hours, the cell viability up to 90% were showed at the concentration between 1 to 10 μg/mL curcumin treated PBMC. The effect of curcumin on oxygen radical-generated DNA damage in normal cord blood PBMC was investigated by comet assay. The activity of curcumin to protect oxidative stress and apoptosis was presented in this study. The Fenton reaction ($FeSO_4$ and H_2O_2) was used to produce ROS (ex. $OH^{\bullet}$) then $OH^{\bullet}$ induced oxidative stress to cells. When curcumin was cotreated cells with Fenton reaction, the results showed that small protective effect of curcumin at low dose (5, 10 and 15 μg/mL) and protected at dose 20 μg/mL. The result of this present study suggests that curcumin could protect oxidative stress by dose dependent manner of curcumin.

In this study we showed that the comet assay could be used to give reproducible results in estimating the extent of oxidative DNA damage to cells and apoptosis. It thus proved possible to rank the potency of the antioxidant agents tested with high confidence. It was clear that curcumin well recognized as a dietary antioxidant, provides definite protection against oxidative stress in the vitro.

In conclusion, this study showed that the comet assay could be used to give reproducible results in estimating the extent of oxidative DNA damage to cells and apoptosis. Apoptosis in NRBCs of β-thalassemic patients was observed while PBMCs sensitive on iron over load ($FeSO_4$) with H_2O_2 than normal cord blood. Oxidative stress to cells by Fenton reaction could induce cell death *via* apoptosis. It was clear that curcumin well recognized as a dietary antioxidant, provides definite protection against oxidative stress at a dose dependent manner of curcumin *in vitro*.