รายงานวิจัยฉบับสมบูรณ์

โครงการ ผลของเคอร์คูมินต่อความเป็นพิษของรูปแบบแฮปโตโกลบินในธาลัสซีเมียที่เกี่ยวข้องกับภาวะเบาหวาน

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สิงหาคม 2563

MRG5580027

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

Excessive hemoglobin (Hb) enhances production of free radicals *via* Haber Weiss-Fenton reactions. Haptoglobin (Hp) is a cellular protective protein which protect cells from heme iron toxicity. Hp polymorphism has been proposed as a risk factor for developing diabetic mellitus and atherosclerotic vascular disease. Patients carrying Hp 2-2 phenotype are at risk for developing coronary artery disease. We investigated the pathophysiologic mechanisms of heme iron derived from Hb, glycated Hb (Hb(gly)) and Hp-Hb complex. HepG2 cells were treated with various forms of heme iron. labile iron pool (LIP) was measured using calcein-AM technique. Reactive oxygen species (ROS) was investigated using 2,7-dichlorofluorescein and the amount of redox active chelatable iron associated with Hb and Hp-Hb complex was assessed by fluorometric assay with dihydrorhodamine. HDL-associated lipid peroxide was determined using spectrophotometric method. Free Hb and Hb(gly) significantly increased amounts of LIP, ROS, redox active chelatable iron and lipid peroxide. Cells treated with Hb(gly) showed higher levels compared to Hb treated cells. As a part of Hp-Hb complex, Hp stabilized heme iron in heme pocket of Hb and prevented cells from oxidative damage. It was found that Hp 1-1 was superior in reduction of LIP, ROS, redox active chelatable iron and lipid peroxide levels as compared to Hp 2-2. Interestingly, curcumin (25-100 ng/ml) resulted in dramatically decreased heme iron-mediated oxidation. In comparison, curcumin showed a greater decrease in Hp 2-2 treated cells than Hp1-1. These result provide evidence that heme iron, especially, Hb(gly) associated Hp 2-2-phenotype is more redox active further to increase oxidative stress. However, the use of curcumin has the potential to reduce this oxidative damage

Keywords: haptoglobin, hemoglobin, curcumin, oxidative damage, labile iron pool, reactive oxygen species, redox active chelatable iron, lipid peroxide

Introduction to the research problem and its significance

Under increased red cell hemolysis in β-thalassemia a patient, excessive hemoglobin (Hb) is released into plasma and becomes highly toxic substance This hemoprotein can enhance production of free radicals *via* Haber-Weiss-Fenton reactions**.** Under physiological condition, free Hb is captured by haptoglobin (Hp) to form a Hb-Hp complex and the resulting complex is directed to CD163-expressing macrophages and further internalized in Kuffer cells in the liver. This mechanism prevents cells from toxic free Hb. There are many evidences illustrate that iron overloaded-β-thalassemic patients are relate with diabetes (1-3). Frequency of diabetic mellitus (DM) in patients with clinical hemochromatosis ranges in 20-50% (4-7). The main pathophysiologic mechanism that leads to diabetes in hereditary hemochromatosis is thought to involve β-cell dysfunction due to iron toxicity resulting in relative decrease of insulin secretion (8). Iron overload is thought to have direct toxic effects on β-cells in the pancreas. Importantly, patients with β-thalassemia and DM are more prone to develop cardiomyopathy which this serious complication is a major cause of mortality (9).

Hyperglycemia plays an important role in the pathogenesis of long-term complications and further affects vital organs such as heart, nervous system, kidneys and blood vessels. Protein glycation is thought to be a major cause of diabetic pathologies which it can form advanced glycation end-products (AGEs). Glucose can undergo autoxidation in the present of transition metals, especially iron and copper. Reactive oxygen species (ROS) is generated at multiple steps during this process and more generated in thalassemic patients with iron

overload. Recent interest has focused on strategies to prevent, reverse or retard this glycoxidation in order to improve thalassemic-diabetic cardiovascular complications.

Hp is a cellular protective protein which functions in the protection of cells from heme iron toxicity. However, it might be a genetic modifier of *Hfe*-associated hemochromatosis. Hp(2-2) type was over-expressed not only in iron overloaded patients but also in type 2 diabetes mellitus (type 2 DM) or noninsulin dependent diabetes mellitus (NIDDM). It may be exaggerated in diabetic stage given the glycosylation of hemoglobin molecule. More recently, Hp polymorphism has been proposed as a risk factor for developing atherosclerotic vascular disease. Patients carrying Hp(2-2) phenotype are at risk for developing premature coronary artery disease whereas Hp(1-1) phenotype has no risk of myocardial infarction. As mentioned above, myocardial complication is a major cause of death in the β-thalassemic patients. Many researchers have proposed that it is a consequence of nonheme iron catalyzed oxidative damage and involved in lipoprotein functions. In addition, heme is known to exhibit peroxidase-like activity that can oxidize many biomolecules. However, the mechanisms responsible for cardiovascular complication in severe hemolytic-β-thalassemia and diabetes which involved in heme iron, Hb and Hp phenotype have not been completely defined.

Furthermore, inhibition of heme iron-induced toxicity would provide a therapeutic strategy to prevent the cardiovascular diseases in diabetic thalassemic patients. Therefore, the inspection of compounds which could ameliorate above complications is necessary and should be encouraged. In this study, we will investigate the pathophysiologic mechanisms of

heme iron derived from non-glycated Hb, glycated Hb, Hp-bound Hb (both Hp(1-1) and Hp(2-2) phetotypes) *ex vivo* and further study the protective effects of curcumin, best-studied natural compound, in these events.

Literature review

Beta-thalassemia is caused by any of more than 200 point mutations and rarely by deletions. Thalassemia is clinically heterogeneous because of various genetic lesions (10). Hemoglobin-stabilizing protein is a protein that can bind to and stabilize free chains, thereby blocking the production of (ROS) and reducing oxidative damage to erythrocytes. This protein appears to modulate the clinical picture of β-thalassemia in murine model (11) but has not been found to modify in thalassemia patients. Increased red cell hemolysis commonly occurs in thalassemia. The complex chain of α-globin occurs in erythrocytes resulting in their accelerated peripheral destruction. Ineffective erythropoiesis in β-thalassemia causes severe anemia, increased erythrocyte turnover, excessive iron absorption, and eventually iron overload in the liver, heart and endocrine organs (12). With increasing iron overload, transferrin is increasingly saturated 85-105 % and non-transferrin bound iron (NTBI) is detectable 2.7-7.1 μM in plasma compartment (13). NTBI would be a potential chemical catalyst that can generate free radical intermediates and enhance lipid peroxidation of cell membranes and lysosomes (14, 15).

Following red blood cell destruction in β-thalassemia excessive intracellular hemoglobin is released into plasma and acts as a highly toxic substance instead of gas

transportation. This toxicity arises from the heme iron which can produce free radicals *via* Fenton like mechanism (**Fig. 1**)

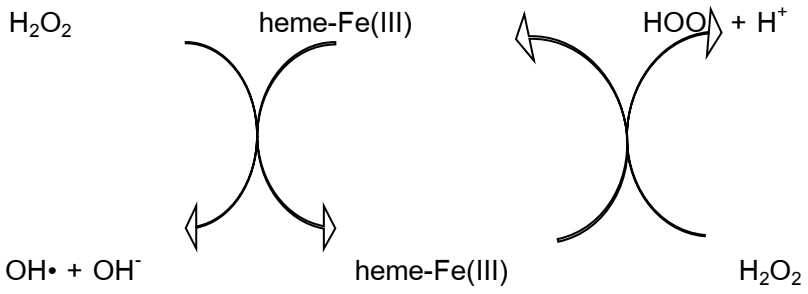


Fig. 1 A Fenton like mechanism which is catalyzed by heme iron.

Under normal conditions, when hemoglobin (Hb) is released from erythrocytes, plasma protein called haptoglobin (Hp) captures the released hemoglobin to form a Hb-Hp complex. The resulting complex is directed to CD163-expressing macrophages and subsequently internalized in hepatic Kuffer cells. In the Kuffer cells, globin chain is hydrolyzed in lysosomes whereas heme moiety is oxidatively degraded by microsomal heme oxygenase (HO) to produce biliverdin, carbon monoxide and iron. One part of the released iron is incorporated into cellular ferritin for storage and the other part is bound to circulating apotransferrin for re-utilization. . Thereby, Hp protein represents the primary defense mechanism against free Hb.

Hp is an α2-sialoglycoprotein with Hb-binding capacity which can capture free Hb to prevent both iron loss via kidney filtration and protect kidney damage. It is characterized by molecular heterogeneity with 3 major phenotypes: Hp(1-1), Hp(2-2) and the heterozygous Hp(2-1). Although Hp is found in serum of all mammals, this polymorphism exists only in humans. Importantly, it has the highest binding affinity for Hb (Kd = 1 pmol/L) (16). Releasing of Hb into plasma is a physiological phenomenon associated with intravascular hemolysis during destruction of senescent erythrocytes and enucleation of erythroblasts. Rapid formation of the Hp-Hb complex reduces glomerular filtration of Hb and limits the loss of iron in urine. In addition, Hp is believed to reduce loss of Hb through the glomeruli, thereby protecting against peroxidative kidney injury and allowing heme iron recycling. The increased susceptibility to Hb- driven lipid peroxidation demonstrated in conditions of hypohaptoglobinemia or anhaptoglobinemia in human and Hp-deficiency mice (17-19). The primary site of Hp expression is liver (20). In β-thalassemia, free radicals also generate hemolysis and further hemolytic anemia. During excessive hemolysis, the Hp- CD163-mediated scavenging mechanism is saturate. The outcome is pathological state with heme-mediated oxidative damage. Circulating amounts of Hp in normal human serum is about 0.45-3 mg/ml (20). Low Hp level is a clinical marker of intravascular hemolysis whereas high Hp level is also a clinical marker of acute phase response.

The Hp protein found in most mammals consists of 2 (αβ)-units linked by disulfide bond between a cysteine of the 2 α-chains to form (αβ)-dimer (21, 22). The main function

of the Hp α-chain appears to be connection of (αβ)-unit to form dimers (Hp(1-1) and Hp(2-2) phenotypes) and multimer (Hp(2-1) phenotype), whereas the interaction with Hb and CD163 is mediated by α-chain (23). The complex formation between Hp and Hb is noncovalent interaction with variations in binding strength of the Hp phenotypes (Hp(1-1)>Hp(2-1)>Hp(2-2)) (24). In the opposite, CD163 exhibits a higher functional affinity for the complexes generated between Hb and Hp(2-2) than it does for Hp(1-1)-Hb complex (25). Somewhat paradoxically to this higher affinity for CD163, Hp(2-2)-Hb has been reported to exhibit a lower rate of CD 163-mediated clearance than the complexes formed between Hp(1-1) and Hb (26). The capacity to protect against Hb-mediated oxidation also appears to differ between phenotypes. Hp(1-1) demonstrates superior antioxidant capacity compared with Hp(2-2) (27).

Dietary macronutrients, fats and carbohydrate are well established factors in etiology of diabetes (28) but little is known about the role of micronutrients in relation to diabetes risks. The increase of total body iron stores was reported to be associated with an increased risk of the development of type 2 diabetic mellitus (29-31). Altered iron homeostasis has been reported in rat models of diabetes (32). Patients with type 2 diabetes have higher circulating ferritin levels compared to non-diabetic individuals (33). Serum ferritin levels positively correlate with levels of circulating insulin and glucose (34-36), hypertension (34, 37), dyslipidemia (34, 38) and obesity (34, 39) it also relates with pathological conditions such as the metabolic syndrome (40), gestational diabetes (41) and cardiovascular disease

(42). Frequency of DM patients with clinical hemochromatosis range in 20-50% (4-7). The main pathophysiologic mechanism that leads to diabetes in hereditary hemochromatosis is thought to involve β-cell dysfunction due to iron toxicity resulting in relative decrease insulin secretion (8). Iron overload is thought to have direct toxic effects on β-cells. *Hfe*$^{-/-}$ mice demonstrated decreased insulin content and increased in markers of apoptosis. Islet perfusion experiments demonstrated a decreased in islet cell sensitivity for insulin secretion in response to glucose (8). One possible mechanism of iron-induced toxicity is the promotion of oxidative stress which was increased by 58% in *Hfe*$^{-/-}$ islets as compared to *Hfe*$^{+/+}$ (43).

A previous study shows that among 167 Cys282Tyr homozygous patients, a low *Hp1* allele frequency (0.30) was found whereas there was overrepresentation of the Hp(2-2) type (49%), suggesting that Hp(2-2) phenotype is associated with an accelerated iron overload. Male patients carrying Hp(2-2) had higher serum ferritin levels than those with a Hp(1-1) or Hp(2-1) (44). It has been proposed that Hp might be genetic modifier of *Hfe*-associated hemochromatosis as Hp(2-2) type was overexpressed in hemochromatotic patients and iron loading was more pronounced in patients carrying Hp(2-2) (44). In the recent, research has shown that the Hp genotype may characterize susceptible individuals with type 2 DM. Specifically, Hp is proposed to have special relevance in diabetic mellitus as these differences may be exaggerated in diabetic stage given the glycosylation of hemoglobin molecules.

Patients with thalassemia and diabetes mellitus are more prone to develop cardiomyopathy (9). This complication is serious and is a major cause of mortality. The pathophysiology of

cardiomyopathy in β- thalassemia and DM is incompletely understood and several mechanistic approached are under debate.

Recently, Hp polymorphism has been proposed as a risk factor for developing atherosclerotic vascular disease, independent of the classical risk factors such as smoking, hypertension, diabetes mellitus and lipid concentrations. Males carrying Hp2-2 phenotype are at risk for developing premature coronary artery disease (45). Patients with the Hp(1-1) phenotype had no risk of myocardial. In the contrast, patients carrying the Hp2-2 phenotype are at risk for developing premature coronary artery disease (45). In addition, diabetic patients who are homozygous for the Hp1 allele are provided increased protection against the development of diabetic vascular complications (46).

Several mechanisms have been proposed to explain the harmful effects of hyperglycemia. Glucose could be directly toxic to cell components because it can undergo autooxidation and generate $OH^{\bullet}$ radicals (47). It can react with protein in non-enzymatic manner leading to development of Amadori products followed by the formation of AGE. ROS is generated at multiple steps during this process and more generated by the presence of transition metal, especially in iron-overloaded thalassemic patients. Recent interest has focused on strategies to prevent, reverse or retard this glycoxidation in order to improve thalassemic-diabetogenic cardiovascular complications.

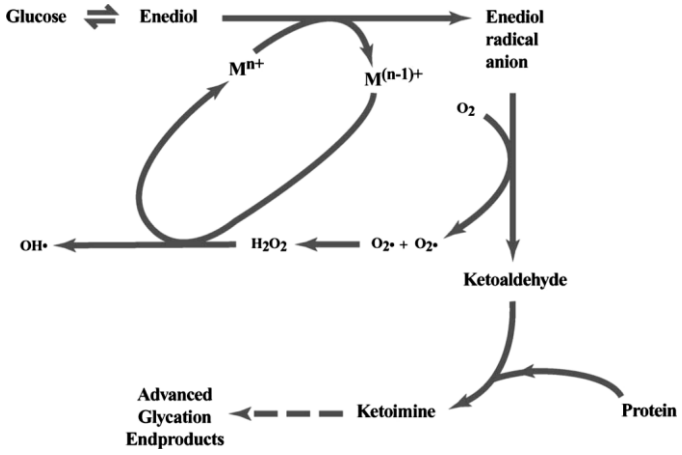


Fig. 2 Glycooxidation produces reactive oxygen species (47).

Curcumin (diferuloylmethane) is the yellow pigment in curry from turmeric. It is one of the best-studied natural compounds which shown many beneficial effects, including anti-inflammatory, anti-oxidative stress, anti-viral, anti-hypercholesterolemic, anti-infective and anti-carcinogenic effects. Curcumin treatment at 12 weeks could exert beneficial effect in diabetes. It improved pancreatic islets by neogenesis characterized by the presentation of small islets increase in numbers nearly the duct (48). Rungseesativanon and her colleagues showed that curcumin supplementation could improve diabetes-induced endothelial dysfunction (49) and also inhibit oxidized LDL-induced hepatic fibrosis (50). Curcumin and its derivatives are capable of scavenging free radicals, chelating and disarming oxidative metal ions (51). Biological activities of the CUR have been attributed to hydroxyl group substituted on the benzene rings and diketonic structure, which the latter can undergo a keto-

enol tautomerisation (51, 52). Curcumin bound iron to produce a Fe^{3+}-curcumin complex with a formation constant of 10^{22} (52, 53). The strong chelating ability of diketones has been widely investigated towards many metal ions. Therefore, CUR could be of great importance in treatment of iron overload and alleviation of oxidative stress. Our group demonstrated that curcumin bound Fe(III) ion in solution and chelated NTBI in β-thalassemic serum efficiently (54). It is hypothesized that curcuminoids would adopt the diketone group as iron-binding sites to bind and remove the iron distributed in plasma compartment and vital organs. Recently, we demonstrated that curcumin could effectively chelate toxic non-heme iron in plasma and vital organs of iron-loaded thalassemic mice. Consequently, it can alleviate non-heme iron toxicity and harmfulness of free radicals (55). Moreover, we elucidated that curcumin supplement improved the depressed heart rate variability (cardiac autonomic disturbance) in iron-loaded thalassemic mice (56).

Although, there is a number of researches elucidated curcumin ameliorated nonheme iron induced pathologies in severe hemolytic-β-thalassemia and diabetes, the mechanism underlying toxicity of heme iron and Hp polymorphisms are not fully understood. In this study, we will investigate the pathophysiologic mechanisms of heme iron derived from non-glycated Hb, glycated Hb, Hb bound with Hp (both Hp(1-1) and Hp(2-2) phetotypes) *ex vivo*. Moreover, a possible target for the management of multiple risk factors in cardiovascular complications in diabetic-β-thalassemic patients could be the prevention or inhibition of heme iron induced

toxicity. Therefore we will further investigate the protective effects of curcumin on these events.

Objectives

1. To study the role of excessive hemolysis on cardiovascular complications in diabetes mellitus related β-thalassemia.

2. To investigate the pathophysiologic mechanism of Hb, Hb_{gly} and Hp polymorphisms in diabetes mellitus related β-thalassemia.

3. To examine the effect of curcumin on Hb, Hb_{gly} and Hp polymorphism-induced diabetes mellitus related β-thalassemia complications.

Method

1. Preparation of hemoglobin

Hemoglobin will be isolated from fresh human blood. Red blood cells will be washed five times with phosphate buffer saline (PBS), pH 7.0 and lysed with ice cold water. Hemolysate will be separated by centrifugation at 15,000 g for 15 minutes.

2. Preparation of glycated hemoglobin

Glycated Hb will be freshly prepared for each experiment using glycoaldehyde. Hemoglobin (10 mM) will be incubated with 25 mM glycoaldehyde, 1.5 mM phenylmethylsulfonyl fluoride (PMSF), 1% pencillin-streptozocin and 40 μg/ml gentamicin for 3 days at 37 °c. Glycated Hb will be dialyzed in PBS for 48 hours.

3. Preparation of high density lipoprotein

High density lipoprotein (HDL) will be isolated by a rapid sedimentation ultracentrifugation method. The background density of 1.2 ml of normal plasma in 3 ml ultracentrifuge tube will be adjusted to d = 1.063 kg/l by the addition of solid potassium bromide (KBr). The density adjusted plasma will be gently overlaid with an aqueous solution of KBr (d = 1.063 kg/l). Then, the ultracentrifugation will be performed using fixed-angle rotor at 541,000 g_{max} at 48°c for 120 minutes. The supernatant containing VLDL and LDL will be

removed by tube slicing at 1.2 cm and will be aspirated in a volume of 1.8 ml. The remaining infranatant corresponds to HDL and will be recovered in volume of 1.2 ml.

4. Measurement of redox active chelatable iron associated Hb and Hp-Hb complex

The amount of redox active chelatable iron associated with Hp-Hb will be measured using the method of Cabanchik *et al*. (57) with slightly modification. Dihydrorhodamine (DHR) will be used as a sensitive fluorescent indicator of oxidative activity. The iron-dependent component of DHR oxidation by Hb or Hb_{gly} or (Hp1-1)-Hb or (Hp1-1)-Hb_{gly} or (Hp2-2)-Hb or (Hp2-2)-Hb_{gly} will be determined by monitoring the oxidation of DHR in the presence and absence of deferiprone (DFP). Quadruplicates of 20 ml of Hb or Hb_{gly} or (Hp1-1)-Hb or (Hp1-1)-Hb_{gly} or (Hp2-2)-Hb or (Hp2-2)-Hb_{gly} will be transferred to 96-well plates. Two wells will be added with with 180 μl of iron-free HEPES buffer saline containing 50 μM DHR and 2.5 μM ascorbate. The other two wells will be added with the same solution containing 40 μM of DFP. Immediately following the addition of reagent, the kinetics of fluorescence increase will be followed at 37°c in a spectrofluorometer with 485/538 nm for 40 minutes, with readings every 2 minutes. The slopes of DHR fluorescence intensity with time will be determined from measurements taken between 15-40 minutes. The free redox active iron concentration (in μM) will be calculated from calibration curves relating the difference in slope with and without chelator versus iron concentration. Calibration curves will be obtained by spiking plasma-like

media or HEPES-buffer saline (HBS) with ferric-nitrilotriacetic acid (Fe^{3+}-NTA) to give a final concentration of 40-100 μM.

5. Effect of curcumin on Hb and Hp-Hb associated redox activity

The effect of curcumin on Hb and Hp- Hb complex induced oxidation will be investigated using redox active chelatable iron as described above (Section 10.4) with slightly modification. Quadruplicates of 20 ml of Hb or Hp or Hp-Hb (nonglycated and glycated oxy- or MetHb and Hp1 and Hb2) will be transferred to 96- well plates. Two wells will be added with with 180 μl of the iron- free HBS containing 50 μM DHR and 2.5 μM ascorbate or various concentrations of curcumin. The other two wells will be added with the same solution containing 40 μM of DFP. Immediately following the addition of reagent, the kinetics of fluorescence increase will be followed at 37°c in a spectrofluorometer with 485/538 nm for 40 minutes, with readings every 2 minutes. The slopes of DHR fluorescence intensity with time will be determined from measurements taken between 15- 40 minutes. The free redox active iron concentration (in μM) will be calculated from calibration curves relating the difference in slope with and without chelator versus iron concentration. Calibration curves will be obtained by spiking plasma- like media or HBS with Fe- NTA to give a final concentration of 40- 100 μM. The ability in oxidation prevention of curcumin will be measured using the increasing DHR oxidation rate compared to reaction without curcumin.

6. Measurement of HDL-associated lipid peroxides

Purified HDL, glycated or non-glycated Hb and 10 μM of hydrogen peroxide will be incubated in PBS in the presence or absence of various concentrations of curcumin overnight at 37°C. The complex of Hp-Hp will be formed by incubation of Hp1-1 or Hp2-2 with Hb in the equal molar ratio at room temperature for 20 min before the complex will be incubated with HDL. Mixture will be incubated overnight. Lipid peroxides will be determined by addition of 1 ml of lipid peroxide reagent which contained 0.2 M potassium hydrogenphosphate, 0.12 M potassium iodide, 0.15 M sodium azide, 2 g/l igepal, 0.1g/l alkylbenzl-dimethyl ammonium chloride and 10 μM ammonium molybdate. After incubation in the dark for 45 minutes, the mixture will be centrifuged. Optical density will be determined at 365 nm. The amount of lipid peroxides in nmol will be measured using the extinction coefficient of lipid peroxide.

7. Assay of lecithin: cholesterol acyltransferase (LCAT) activity

Pooled thalassemic or diabetic plasma will be incubated with Hb or Hb_{gly} or (Hp1-1)-Hb or (Hp1-1)-Hb_{gly} or (Hp2-2)-Hb or (Hp2-2)-Hb_{gly} in the presence or absence of curcumin. The LCAT activity will be investigated using fluorometric technique. The principle is non-hydrolyzed LCAT substrate fluoresces at 470 nm. Upon hydrolysis by LCAT, a monomer is released from the LCAT Substrate that fluoresces at 390 nm. LCAT activity is assessed as a change in 470/390 emission intensity.

8. Investigation of globin chain cross-linking

Previous study elucidated that exposing Hb to H_2O_2 resulted in a globin chain polymerization (52). During severe hemolysis, non-Hp-bound Hb is readily filtered by the kidney and it has previously been shown that myoglobin does redox cycle H_2O_2 and lipid peroxides in kidney (58). Thus, we will investigate whether curcumin protects globin chain cross-linking. Hb and Hp-Hb in various ratios will be treated with excess concentration of H_2O_2 for 60 minutes. Globin chain cross-linking will be analyzed with SDS-PAGE and silver staining.

9. Determination of labile iron pool in cultured cells exposed Hb and Hp-Hb

The amount of labile iron pool in HepG2 cells will be determined using calcein-AM technique. Briefly, HepG2 cells will be treated with Hb or Hb_{gly} or (Hp1-1)-Hb or (Hp1-1)-Hb_{gly} or (Hp2-2)-Hb or (Hp2-2)-Hb_{gly} in the presence or absence of various concentrations of curcumin. After incubation, cells will be incubated with 0.15 μM calcein –AM for 10 minutes at 37°c in PBS containing 1 mg/ml bovine serum albumin (BSA) and 20 mM HEPES, pH 7.3. After calcein loading, cells will be trypsinized, washed and re-suspended in 2.2 ml of the same buffer without calcein-AM. Then it will be placed in a stirred, thermostatically controlled at 37°C fluorescence spectrophotometer. The fluorescence will be monitored at λ_{ex} 488 nm and λ_{em} at 518 nm.

10. Investigation of curcumin cytotoxicity

Cell toxicity will be examined in vitro using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye assay (59). Cells ($3x10^3$cells/well) are treated with the tested compounds at 37^oC for indicated time and incubated with MTT dye solution. Resulting purple-colored formazan product will be solubilized with DMSO solvent and measured optical density (OD) at 540/630 nm with a UV/VIS dual-wavelength spectrophotometer. The intensity of the purple color is directly related to the number of viable cells, but does not appear on dead cells.

11. Assessment of reactive oxygen species in cultured cells exposed Hb and Hp-Hb

Toxicity of glycated or non-glycated Hb and Hp-Hb complexes will be investigated using 2,7-dichlorofluorescein (2,7-DCFH). Hepatocytes will be incubated with Hb or Hb_{gly} or (Hp1-1)-Hb or (Hp1-1)-Hb_{gly} or (Hp2-2)-Hb or (Hp2-2)-Hb_{gly} under low (100 mg/dl) or high (450 mg/ml) glucose DMEM for 16 hours in the presence of various concentrations of curcumin. Cells showing fluorescence will be reflected the amount of reactive oxygen species.

12. Investigation of mRNA expression

HepG2 cells will be treated with Hb or Hb_{gly} or (Hp1-1)-Hb or (Hp1-1)-Hb_{gly} or (Hp2-2)-Hb or (Hp2-2)-Hb_{gly} in the presence or absence of various concentrations of curcumin. Total RNA will be isolated from HepG2 cells using TRIzol reagent at the ratio of 10^6 cells/ml

Trizol reagent. Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) will be performed for investigation of Hemoxygenase-1 (HO-1), transferrin receptor (TfR), ferritin, insulin receptor and scavenger receptor class B type I (SR-BI) mRNA expression.

13. Effects of curcumin on toxicity of Hb and Hp-Hb complex in β-thalassemia and diabetes

As mentioned above, hemochromatotic and diabetic sera represented high Hp2-2 polymorphism. Therefore, we will identify Hp phenotypes in β-thalassemic and diabetic sera using SDS-PAGE followed by peroxidase staining. Pooled β-thalassemic or diabetic sera with high amount of Hp2-2 will be incubated with HepG2 cells in the presence or absence of curcumin. Amounts of labile iron pool, reactive oxygen species and mRNA expressions will be performed as describe above.

14. Statistical analysis

Significant difference between values will be assessed using non-parametric Mann-Whitney U test and all values will be expressed as mean±SD. Statistical significance will be assumed at $p < 0.05$.

Results

1. Cytotoxicity of Hb, Hb(gly), Hp-Hb complex, Hp-Hb(gly) complex and curcumin to HepG2 cells

MTT assay was performed to assess the cell viability activity of Hb, Hb(gly), Hp-Hb complex, Hp-Hb(gly) complex, and curcumin against HepG2 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye assay. Toxicity was not observed after the treatment with Hb, Hb(gly), Hp 1-1/Hb, Hp 2-2/Hb Hp1-1/Hb(gly), and Hp 2-2/Hb(gly) at a concentration of 12.5-400 ug/ml (**Table 1 and Figure 3**). In contrast, HepG2 cell showed dosage-dependent cytotoxicity at a concentration of 0.312-10 ug/mL (**Table 2 and Figure 4**)

Table 1 Evaluation of cell viability by MTT assay on HepG2 cells treated with Hb and Hp-Hb complex. The data are presented as the mean ± standard deviation (SD).

Concentration (nM/ml)	Survival cells (%)							
	Hb	Hb(gly)	Hp1-1	Hp2-2	Hp1-1/Hb	Hp2-2/Hb	Hp1-1/Hb(gly)	Hp2-2/Hb(gly)
0	100±0.03	100±0.00	100±0.05	100±0.05	100±0.08	100±0.06	100±0.08	100±0.06
12.5	94±2.79	91±8.95	92±5.86	95±6.89	97±8.45	90±2.73	90±19.15	90±3.92
25	96±5.01	90±12.54	91±5.17	90±3.10	95±4.71	88±3.61	109±20.01	83±11.65
50	97±3.94	95±4.07	93±5.52	96±5.59	96±6.53	91±2.58	101±15.05	89±0.78
100	96±2.99	102±12.28	95±4.55	96±8.67	95±5.09	92±6.12	100±21.89	100±8.86
200	99±7.61	104±11.58	96±8.17	97±9.14	95±11.31	95±3.53	103±11.65	96±13.94
400	95±2.62	89±7.74	94±5.28	92±7.74	96±7.86	95±8.94	91±17.51	89±6.39

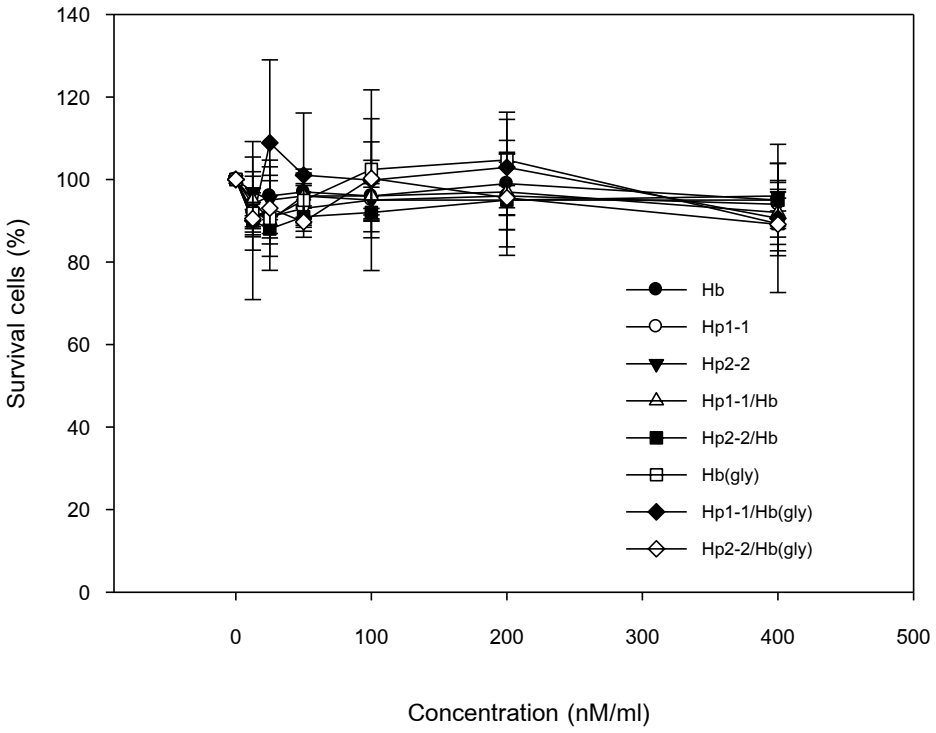


Figure 3 Evaluation of cell viability by MTT assay on HepG2 cells treated with Hb and Hp-Hb complex. The data are presented as the mean ± standard deviation (SD).

Table 2 Evaluation of cell viability by MTT assay on HepG2 cells treated with curcumin. The data are presented as the mean ± standard deviation (SD).

Concentration (nM/ml)	Survival cells (%)
0	100±0.06
0.312	97±4.96
0.625	97±6.07
1.25	102±6.50
2.5	94±8.82
5	82±5.34
10	43±8.01

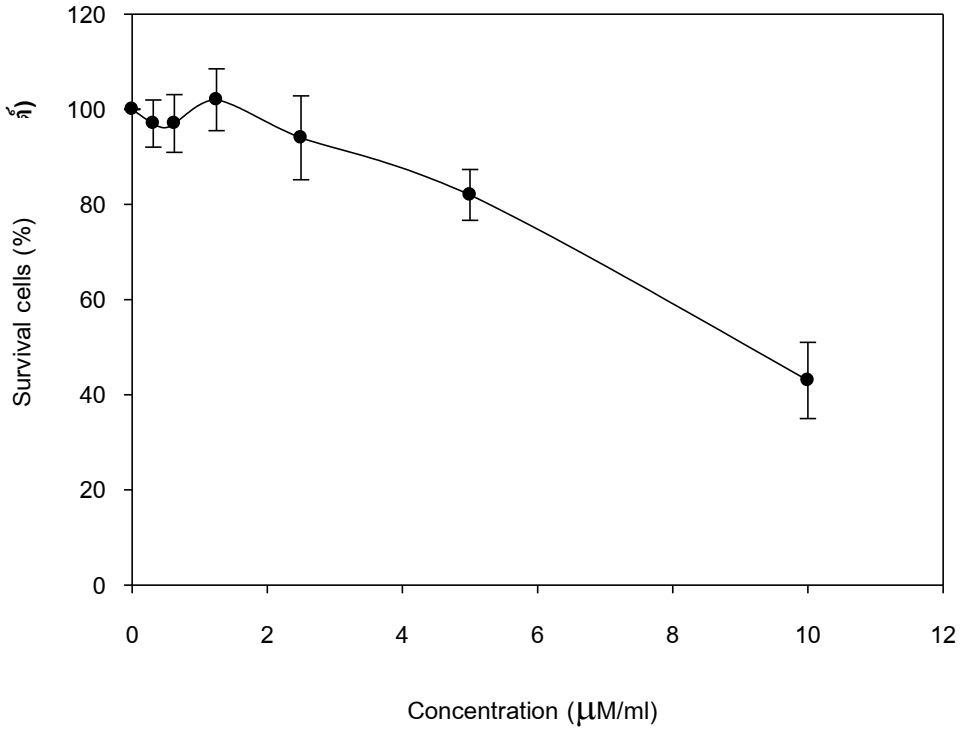


Figure 4 Evaluation of cell viability by MTT assay on HepG2 cells treated with curcumin. The data are presented as the mean ± standard deviation (SD).

2. Effects of Hb, Hb(gly), Hp/Hb complex, Hp/Hb(gly) complex and curcumin on labile iron pool; LIP) in HepG2 cells

HepG2 cells were treated with Hb, Hb(gly), Hp-Hb complex, Hp-Hb(gly) complex, and curcumin to study labile iron pool (LIP) by calcein-AM. The LIP can be quantified based on its ability to bind to calcein-AM. CA-AM becomes fluorescent upon hydrolysis by intracellular esterases in live cells. Its fluorescence is quenched upon binding to LIP. The fluorescence intensity is proportional to the amount of cellular LIP.

The results showed that fluorescence intensity in control group revealed 300.00±15.59 unit. A reduction fluorescence intensity (261.75±12.20 unit) was observed after treatment with Hb at concentration of 1 ug/mL. Theses results showed that high free Hb level increased LIP level. Cells treated with Hp1-1/Hb complex revealed a greater fluorescence intensity when compared to the group treat with only Hb, with increase of 281.50±14.82 unit. These results demonstrated that the binding of Hp1-1 and Hb reduce LIP level, less with Hb alone. Moreover, cells treated with Hp2-2/Hb complex exhibited no obvious association in LIP level in cell.

Cells treated with Hb(gly) at concentration of 1 ug/mL decresed fluorescence intensity of 227.33±23.02 unit, indicating that high free Hb(gly) increased LIP level. Cells treated with Hp1-1/Hb(gly) and Hp2-2/Hb(gly) showed higher fluorescence intensity compared to Hb(gly) treatment group, with increase of 259.66±16.50 and 237.00±28.16, respectively. These finding demonstrated that the Hp1-1 and Hb(gly) binding help in the reduction of LIP

level, lower than those of Hb (gly) treatment. Moreover, Hb(gly) in cell can cause higher LIP level than those of normal Hb level (**Table 3 and Figure 5**).

Table 3 Effect of Hb, Hb(gly), Hp/Hb and Hp/Hb(gly) on labile iron pool (LIP) in HepG2 cells. The data are presented as the mean ± standard deviation (SD).

Hb (1ng/ml)	**Fluorescence intensity (unit)**
Control	300.00±15.59
Hb	261.75±12.20
Hb(gly)	227.33±23.02
Hp1-1/Hb	281.50±14.82
Hp2-2/Hb	259.75±10.21
Hp1-1/Hb(gly)	259.66±16.50
Hp2-2/Hb(gly)	237.00±28.16

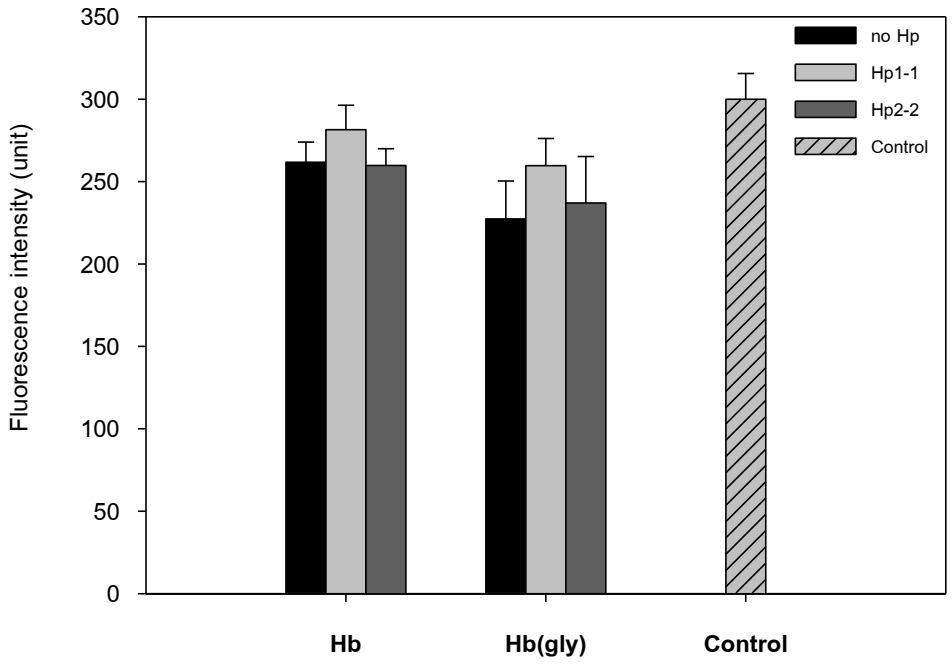


Figure 5 Effects of Hb, Hb(gly), Hp/Hb complex, Hp/Hb(gly) complex and curcumin on labile iron pool; LIP) in HepG2 cells (mean±SD)

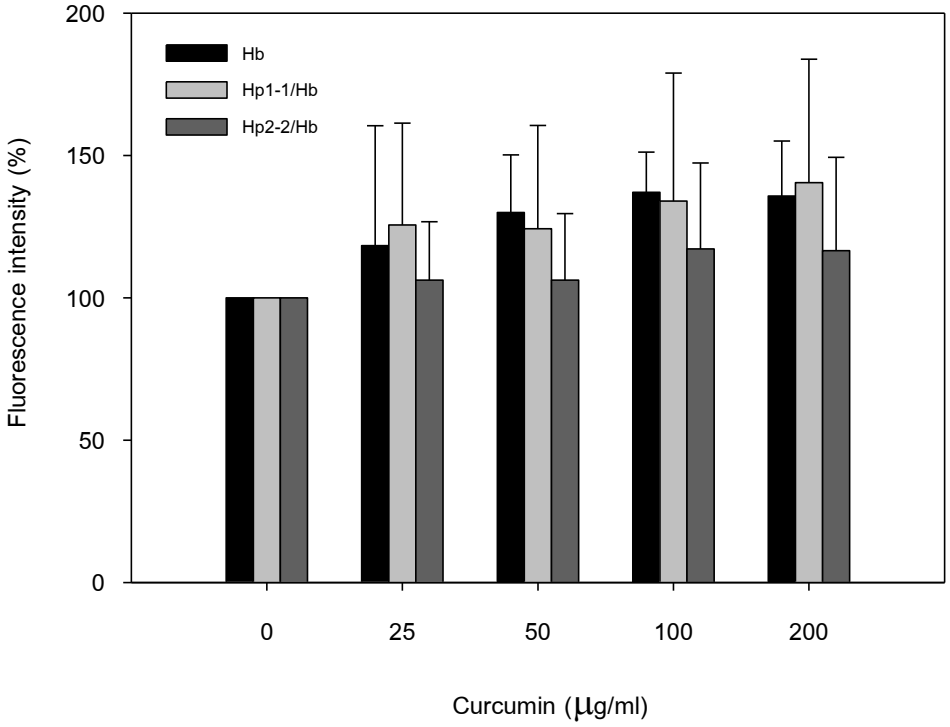
Effects of curcumin on labile iron pool (LIP) in Hb or Hp/Hb – induced HepG2 cells

Fluorescence intensity of curcumin (the range of 25-100 ug/mL) co-tratment with Hb(gly) or Hp1-1/Hb or Hp2-2/Hb at at concentration of 1 µg/mL on HepG2 cells induced by 100 mg/dl glucose was higher in dose dependent manner as compared to control group. These result showed that curcumin can reduce LIP level. Combination of curcumin with Hp2-2/Hb exhibited a reduction level of LIP compared to cells treated with Hb1-1/Hb **(Table 4 and Figure 6)**

Co-treatment of curcumin and Hp2-2/Hb decreased oxidative stress which was higher that those combination with Hb1-1/Hb. Moreover, Cells treated with curcumin and Hp2-2/Hb lowered the LIP level than those with curcumin and Hp2-2/Hb treatment.

Table 4 Effect of curcumin to labile iron pool (LIP) in HepG2 induced with Hb and Hp/Hb at concentration of 1 ug/mL. The data are presented as the mean ± standard deviation (SD)

Curcumin (ng/ml)	**Fluorescence intensity (% of control)**		
	Hb	**Hp1-1/Hb**	**Hp2-2/Hb**
0	100±0.00	100±0.00	100±0.00
25	118.29±42.14	106.17±20.57	125.58±35.79
50	129.95±20.26	106.20±23.39	124.27±36.26
100	137.06±14.11	117.18±30.13	133.97±44.94
200	135.72±19.32	116.55±32.79	140.45±43.38



รูปที่ 6 Effect of curcumin to labile iron pool (LIP) in HepG2 induced with Hb and Hp/Hb at concentration of 1 ug/mL. The data are presented as the mean ± standard deviation (SD)

Effects of curcumin to labile iron pool (LIP) in Hb(gly)-induced HepG2 cells

The fluorescence intensity of curcumin was improved in the presence of Hb(gly) or Hp1-1/Hb or Hp2-2/Hb at concentration of 1 μg/mL in dose dependent manner, indicating that curcumin can lower the level of LIP. Co-treatment of curcumin and Hp2-2/Hb decreased oxidative stress which was higher that those combination with Hb1-1/Hb. (Table 5 and Figure 5).

Table 5 Effect of curcumin to labile iron pool (LIP) in HepG2 induced with Hb(gly) and Hp/Hb(gly) at concentration of 1 ug/mL. The data are presented as the mean ± standard deviation (SD)

Curcumin (ng/ml)	**Fluorescence intensity (% of control)**		
	Hb(gly)	**Hp1-1/Hb(gly)**	**Hp2-2/Hb(gly)**
0	100±0.00	100±0.00	100±0.00
25	108.32±1.80	104.42±10.97	118.31±21.44
50	114.84±3.50	108.40±21.96	130.45±19.40
100	120.29±12.70	117.50±23.36	143.48±20.64
200	89.28±18.35	113.57±33.75	124.90±25.07

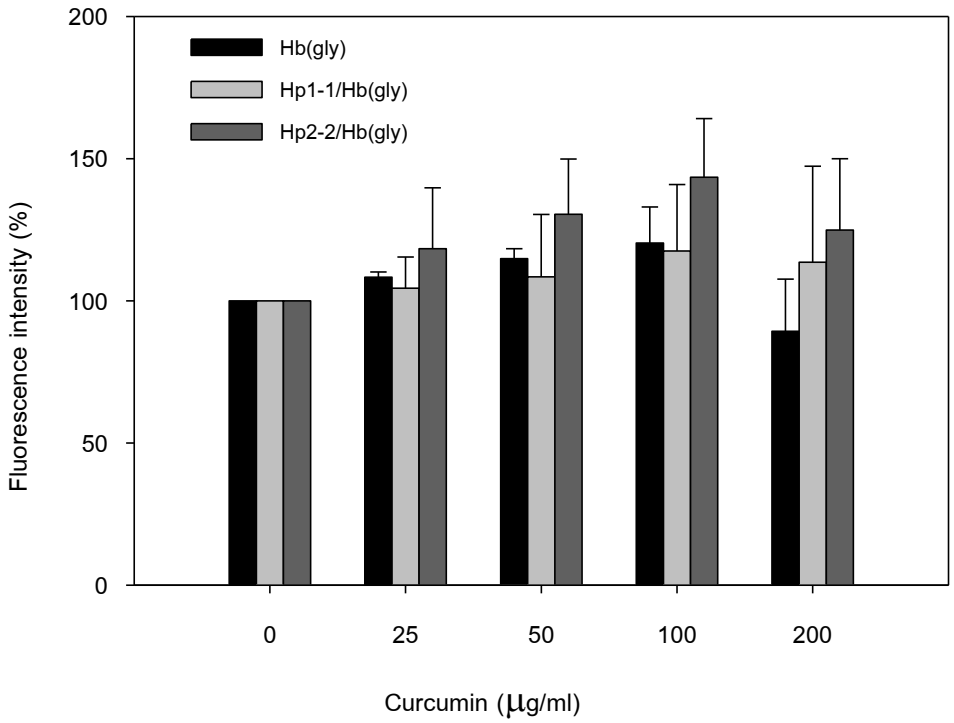


Figure 7 Effect of curcumin to labile iron pool (LIP) in HepG2 induced with Hb(gly) and Hp/Hb(gly) at concentration of 1 ug/mL. The data are presented as the mean ± standard deviation (SD)

Effects of Hb, Hb(gly), Hp/Hb complex, Hp/Hb(gly) complex and curcumin to reactive oxygen species (ROS) in HepG2

HepG2 cells were treated with Hb, Hb(gly), Hp-Hb complex, Hp-Hb(gly) complex with or without curcumin to evaluate ROS level by 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA). DCFH-DA is hydrolyzed by cellular esterases to 2',7'-dichlorodihydrofluorescein (DCFH) and is then oxidixed by intracellular ROS to 2',7'-dichlorofluorescein which emits green fluorescence. DCF fluorecence intensity is propotional ROS level in the cell.

Treated HepG2 cells induced by 100 mg/dl glucose with Hb at concentration of 1 μg/mL increased fluorescence intensity (777.50±37.50unit) compared to control group. Co-treatment of Hp1-1/Hb showed lower fluorescence intensity than those in cells treated with only Hb, indicating that the Hp1-1 and Hb binding supports the reduction of ROS level, lower than those of Hb (gly) treatment. In contrast, combination treatment of Hp2-2/Hb provides an increase in fluoresecence intensity of 864.50±44.50 unit, generatingthe higher level of ROS compared to treated cells with Hb and Hp1-1/Hb

Cells treated with Hb(gly) at concentration of 1 ug/mL exhibited high fluorescence intensity of 1367.00±498.03 unit, indicating that high free Hb(gly) increased ROS level. Cells treated with Hp1-1/Hb(gly) and Hp2-2/Hb(gly) showed higher fluorescence intensity compared with treating with Hb(gly), with increase of 1428.63±596.10 and 1560.25±782.25, respectively. These finding demonstrated that the Hp and Hb(gly) binding did not help in the reduction of

ROS level arising from the induction of Hb (gly) in cells. Moreover, Hb(gly) in cell can cause higher ROS level than those of normal Hb level **(Table 6 and Figure 8).**

Table 6 Effects of Hb, Hb(gly), Hp/Hb complex, Hp/Hb(gly) complex and curcumin to reactive oxygen species (ROS) in HepG2 (mean±SD)

Hb (1ng/ml)	**Fluorescence intensity (unit)**
Control	629.00±2.83
Hb	777.50±37.50
Hb(gly)	1367.00±498.03
Hp1-1/Hb	759.50±43.50
Hp2-2/Hb	864.50±44.50
Hp1-1/Hb(gly)	1428.63±596.10
Hp2-2/Hb(gly)	1560.25±782.25

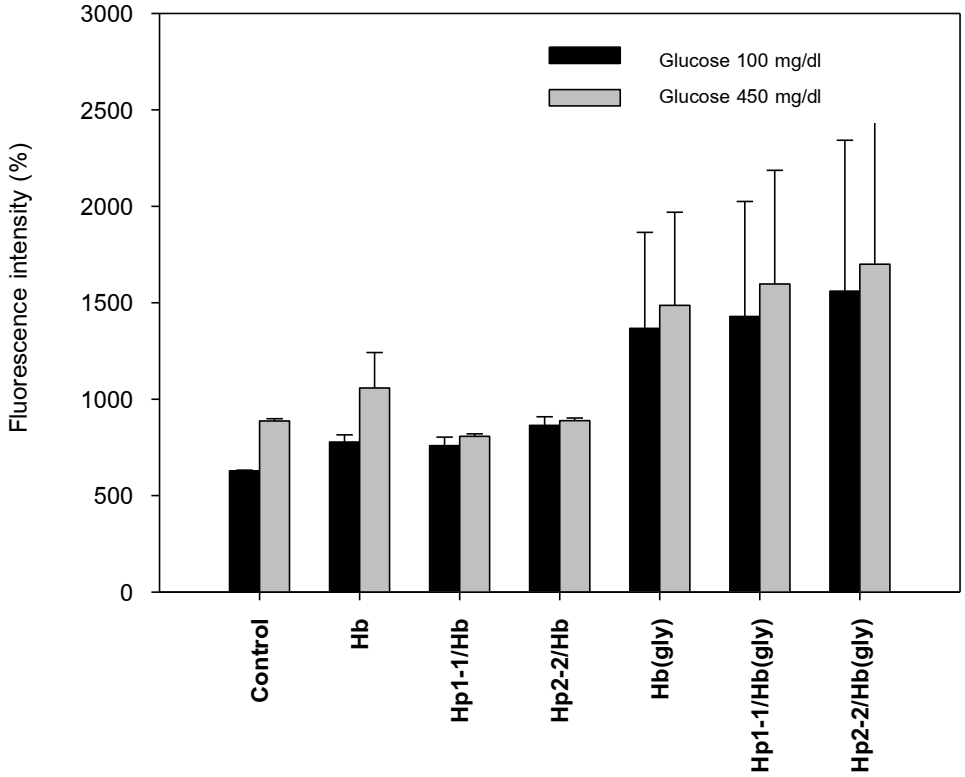


Figure 8 Effect of Hb, Hb(gly), Hp/Hb and Hp/Hb(gly) to ROS in HepG2 cells induced with 100 mg/dl glucose. The data are presented as the mean ± standard deviation (SD)

Effects of curcumin to reactive oxygen species (ROS) in Hb, Hp/Hb – induced HepG2 cells with 100 mg/dl glucose

Curcumin showed a decrease in fluorescence intensity in dose dependent manner (range of 25-100 ug/mL) when co-tratment with Hb or Hp1-1/Hb or Hp2-2/Hb at at concentration of 1 µg/mL on HepG2 cells induced by 100 mg/dl glucose. Co-treatment of curcumin and Hp2-2/Hb decreased oxidative stress which was higher than those of combination with Hb1-1/Hb. Moreover, the reduction of oxidative stress by curcumin supplementation was similar to that of Desferoxamine **(Table 7 and Figure 9)**

Table 7 Effects of curcumin to reactive oxygen species (ROS) in Hb, Hp/Hb at concentration of 1 µg/mL – induced HepG2 cells with 100 mg/dl glucose. The data are presented as the mean ± standard deviation (SD)

Curcumin (ng/ml)	**Fluorescence intensity (% of control)**		
	Hb	**Hp1-1/Hb**	**Hp2-2/Hb**
0	100±0.00	100±0.00	100±0.00
25	79.70±4.81	82.82±3.56	91.47±4.89
50	75.55± 1.71	78.67±5.17	73.53±8.91
100	70.18± 4.48	78.93±1.89	65.30±2.79
200	70.43±0.50	90.46±3.47	86.03±2.01
DFO 20 nM	70.43±0.50	78.99±7.93	87.70±1.94

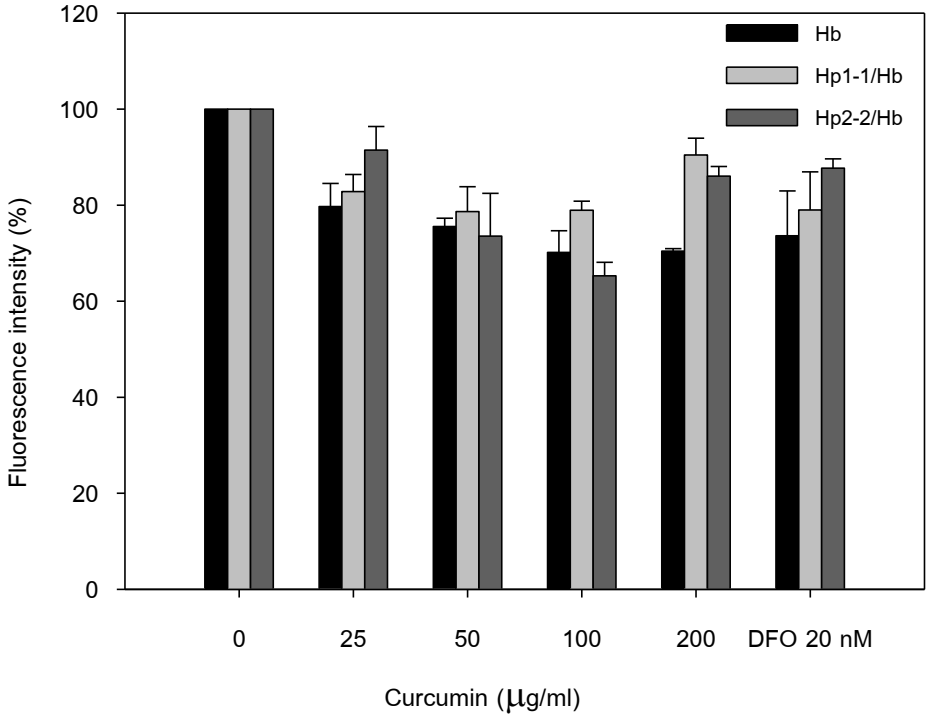


Figure 9 Effects of curcumin to reactive oxygen species (ROS) in Hb, Hp/Hb at concentration of 1 μg/mL – induced HepG2 cells with 100 mg/dl glucose. The data are presented as the mean ± standard deviation (SD)

Effects of curcumin to reactive oxygen species (ROS) in Hb(gly), Hp/Hb(gly) – induced HepG2 cells with 100 mg/dl glucose

Curcumin in the range of 25-100 ug/mL reduced fluorescence intensity when co-tratment with Hb(gly) or Hp1-1/Hb or Hp2-2/Hb at at concentration of 1 µg/mL on HepG2 cells induced by 100 mg/dl glucose. Co-treatment of curcumin and Hp2-2/Hb decreased oxidative stress which was higher that those of combination with Hb1-1/Hb. Moreover, the reduction of oxidative stress by curcumin supplementation was similar to that of desferoxamine **(Table 8 and Figure 10).**

Table 8 Effects of curcumin to reactive oxygen species (ROS) in Hb, Hp/Hb at concentration of 1 µg/mL – induced HepG2 cells with 100 mg/dl glucose. The data are presented as the mean ± standard deviation (SD)

Curcumin (ng/ml)	**Fluorescence intensity (% of control)**		
	Hb(gly)	**Hp1-1/Hb(gly)**	**Hp2-2/Hb(gly)**
0	100±0.00	100±0.00	100±0.00
25	99.50± 5.26	99.31±9.93	98.05± 0.94
50	95.74± 3.35	98.91±5.44	92.45± 3.46
100	94.89±7.40	93.27±4.01	88.83± 1.31
200	99.14±11.78	97.53±1.81	101.10± 6.87
DFO 20 nM	95.33± 12.15	89.74± 15.69	102.46± 3.79

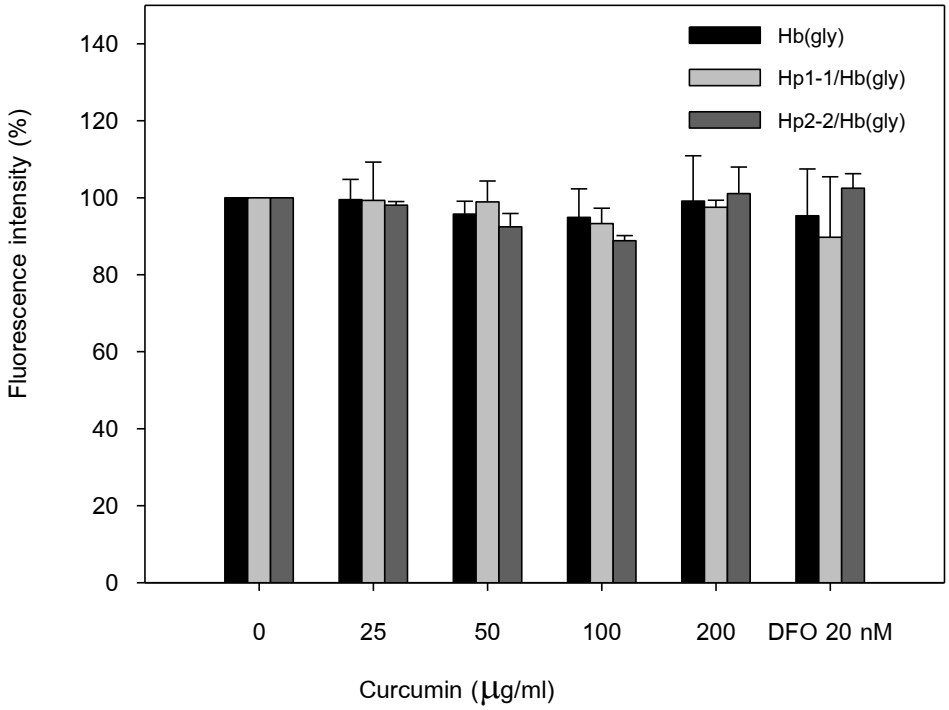


Figure 10 Effects of curcumin to reactive oxygen species (ROS) in Hb, Hp/Hb at concentration of 1 μg/mL – induced HepG2 cells with 100 mg/dl glucose. The data are presented as the mean ± standard deviation (SD)

Effects of Hb, Hb(gly), Hp/Hb complex, Hp/Hb(gly) complex และ curcumin to reactive oxygen species (ROS) in Hb, Hp/Hb – induced HepG2 cells with 450 mg/dl glucose

ROS level was studied in HepG2 cells induced with 450 mg/dl glucose and found that ROS level showed a higher fluorescence intensity in cells exposed to Hb at concentration of 1 ug/mL in compared to control group (from 887.25± 11.32 unit to 1058.00± 184.00 unit), indicating that high free Hb level enhanced the level of ROS. Treated cells with Hp1-1/Hb and Hp2-2/Hb reduce fluorescence intensity, less with Hb alone, with decrease of 807.50±12.50 unit, 888.50±13.50 unit and, 1058.00± 184.00 unit, respectively. These results indicated that the binding of Hp1-1 and Hp2-2 togeter with Hb help in lower the reduction of ROS level than those with Hb alone.

Treated cells with Hb(gly) at concentration of 1 µg/mL exhibited increased fluorescence intensity (1486.12± 483.37 unit) compared to control group (887.25± 11.32 unit), indicating that high free Hb level enhanced the ROS level which did not differ from that of HepG2 cells induced by 100 mg/dl glucose.

Co-treatment of Hp1-1/Hb(gly) and Hp2-2/Hb(gly) showed higher fluorescence intensity than those in cells treated with only Hb(gly), indicating that the Hp and Hb(gly) binding did not help in the reduction of ROS level arising from the induction of Hb (gly) in cells. Moreover, Hb(gly) in cell can cause higher ROS level than those of normal Hb level (Table 9).

Table 9 Effect of Hb, Hb(gly), Hp/Hb and Hp/Hb(gly) to ROS in HepG2 cells induced with 450 mg/dl glucose. The data are presented as the mean ± standard deviation (SD).

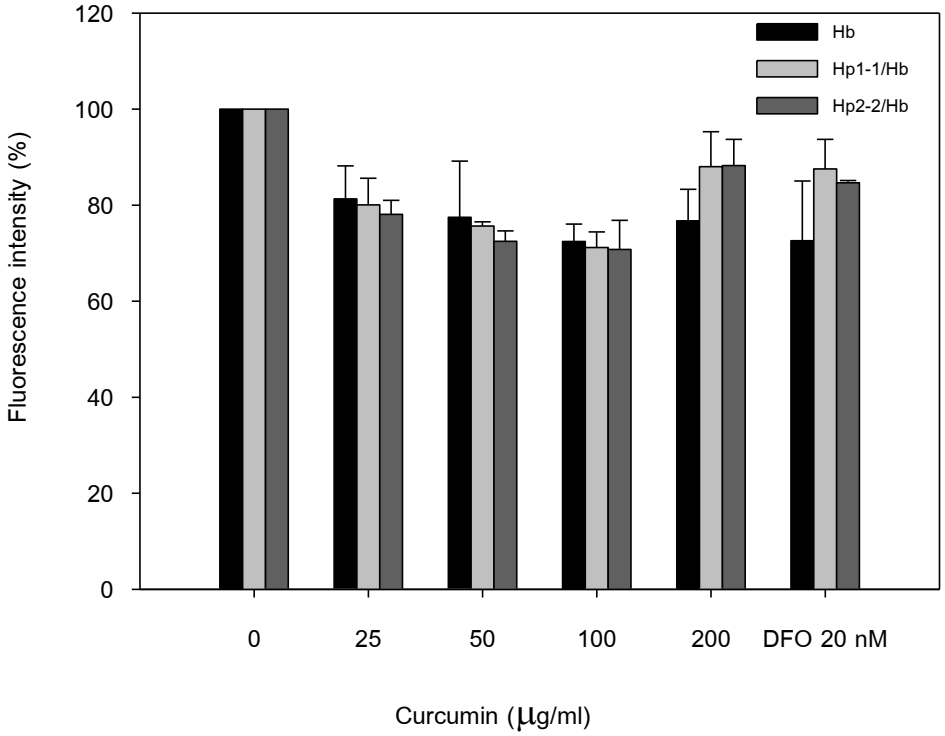
Hb (1ng/ml)	**Fluorescence intensity (unit)**
Control	887.25± 11.32
Hb	1058.00± 184.00
Hb(gly)	1486.12± 483.37
Hp1-1/Hb	807.50±12.50
Hp2-2/Hb	888.50±13.50
Hp1-1/Hb(gly)	1597.625±589.14
Hp2-2/Hb(gly)	1700.13±751.54

Effects of curcumin to reactive oxygen species (ROS) in Hb, Hp/Hb – induced HepG2 cells with 450 mg/dl glucose

Curcumin (concentration range of 25-100 ug/mL) reduced the fluorescence intensity in when co-tratment with Hb(gly) or Hp1-1/Hb or Hp2-2/Hb at at concentration of 1 µg/mL on HepG2 cells induced by 450 mg/dl glucose. Co-treatment of curcumin and Hp2-2/Hb decreased oxidative stress which was higher that those combination with Hb1-1/Hb. Moreover, curcumin efficacy to reduce oxidative stress was similar to that of desferoxamine **(Table 10 and Figure 11).**

Table 10 Effects of curcumin to reactive oxygen species (ROS) in Hb, Hp/Hb at concentration of 1 µg/mL – induced HepG2 cells with 450 mg/dl glucose. The data are presented as the mean ± standard deviation (SD).

Curcumin (ng/ml)	**Fluorescence intensity (% of control)**		
	Hb	**Hp1-1/Hb**	**Hp2-2/Hb**
0	100±0.00	100±0.00	100±0.00
25	81.30± 6.88	80.06± 5.54	78.08± 2.93
50	77.48± 11.70	75.65± 0.87	72.46± 2.16
100	72.43± 3.63	71.18± 3.25	70.76± 6.08
200	76.78± 6.55	88.00± 7.30	88.24± 5.45
DFO 20 nM	72.61± 12.44	87.52± 6.19	84.65± 0.50



รูปที่ 11 Effects of curcumin to reactive oxygen species (ROS) in Hb, Hp/Hb at concentration of 1 μg/mL – induced HepG2 cells with 450 mg/dl glucose. The data are presented as the mean ± standard deviation (SD).

Effects of curcumin to reactive oxygen species (ROS) in Hb(gly), Hp/Hb(gly) – induced HepG2 cells with 450 mg/dl glucose

Curcumin in the range of 25-100 ug/mL reduce fluorescence intensity when co-tratment with Hb(gly) or Hp1-1/Hb(gly) or Hp2-2/Hb(gly) at at concentration of 1 µg/mL on HepG2 cells induced by 450 mg/dl glucose. Combination of curcumin and Hp2-2/Hb(gly) treatment led to reduced oxidative stress which was higher that those combination with Hb1-1/Hb(gly). Moreover, oxidative stress reduction of curcumin was similar to that used by desferoxamine **(Table 11 and Figure 12).**

Table 11 Effects of curcumin to reactive oxygen species (ROS) in Hb(gly), Hp/Hb(gly) at concentration of 1 μg/mL – induced HepG2 cells with 450 mg/dl glucose. The data are presented as the mean ± standard deviation (SD).

Curcumin (ng/ml)	**Fluorescence intensity (% of control)**		
	Hb(gly)	**Hp1-1/Hb(gly)**	**Hp2-2/Hb(gly)**
0	100±0.00	100±0.00	100±0.00
25	96.25±2.67	88.41±6.92	85.00±1.32
50	92.45±6.80	83.59±6.60	82.44±7.76
100	87.63±3.87	79.51±5.12	79.59±5.42
200	95.94±3.17	87.46±8.61	83.50±9.99
DFO 20 nM	92.96±6.50	89.78±9.80	82.36±5.61

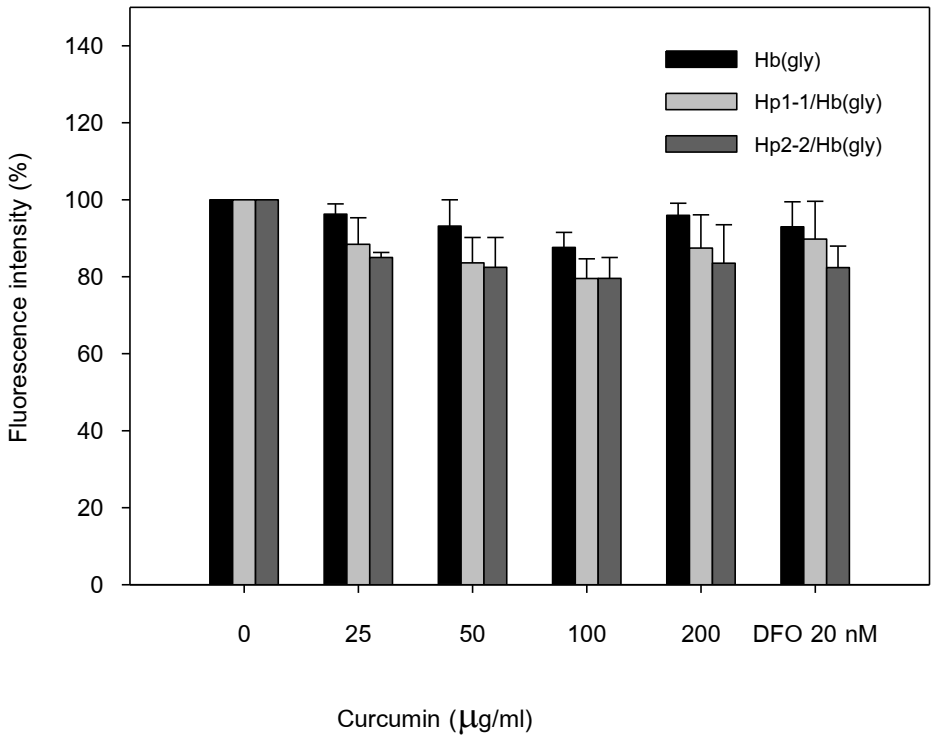


Figure 12 Effects of curcumin to reactive oxygen species (ROS) in Hb(gly), Hp/Hb(gly) at concentration of 1 μg/mL – induced HepG2 cells with 450 mg/dl glucose. The data are presented as the mean ± standard deviation (SD).

Effects of curcumin on redox active chelatable iron in HepG2 cells induced Hb or Hp/Hb

Cells were treated with Hb, Hp1-1/Hb and Hp2-2/Hb at concentration of 1 μg/mL. HepG2 cells treated with Hb revealed highest intracellular redox activity at 35.48±4.99 unit/min. In comparison to only Hb(gly)treatment, the combination treatment of Hp1-1/Hb or Hp2-2/Hb declined oxidation rate at 31.01±6.81 and 33.21±9.25 unit/min, respectively. Combined treatment of curcumin (concentration ranged of 1-4 μg/mL) together with Hb or Hp1-1/Hb or Hp2-2/Hb displayed a reduction in oxidation rate in a dose-dependent manner.

Table 12 Effect of curcumin treatment on oxidation rate in HepG2 induced Hb and Hp/Hb at concentration of 1 μg/mL. The data are presented as the mean ± standard deviation (SD).

Curcumin (μg/ml)	Oxidation rate (unit/min)		
	Hb	Hp1-1/Hb	Hp2-2/Hb
0	35.48±4.99	31.01±6.81	33.21±9.25
1	33.93±5.58	29.49±8.02	30.38±6.50
2	31.52±5.28	27.26±7.64	27.98±6.51
4	30.39±6.02	25.87±6.35	26.31±7.07
L1 1 mM	5.85 ± 0.77	5.10±0.84	4.81±1.79

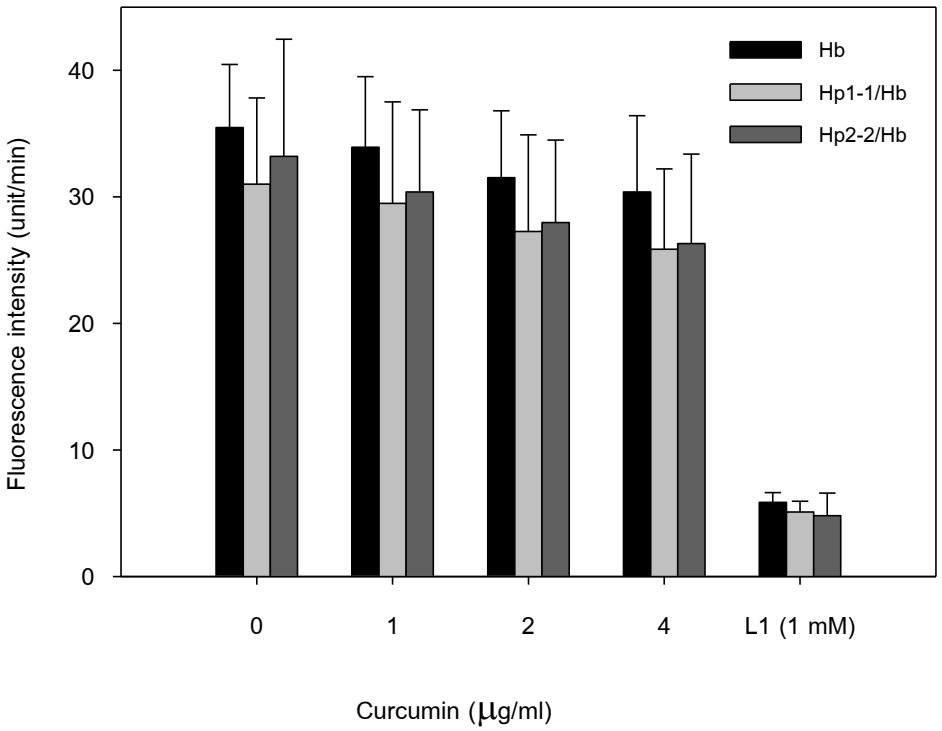


Figure 13 Effect of curcumin treatment on oxidation rate in HepG2 induced Hb and Hp/Hb at concentration of 1 μg/mL. The data are presented as the mean ± standard deviation (SD).

Effects of curcumin on redox active chelatable iron in HepG2 cells induced Hb or Hp/Hb(gly)

Cells were treated with Hb(gly), Hp1-1/Hb(gly) and Hp2-2/Hb(gly) at concentration of 1 μg/mL. HepG2 cells treated with Hb(gly) exhibited highest intracellular redox activity at 32.72±5.80 unit/min. The combination treatment of Hp1-1/Hb(gly) decreased oxidation rate at 30.69± 5.27 unit/min in comparison to only Hb(gly) treatment. On the other hand, the combination treatment of Hp2-2/Hb(gly) complex slightly increased the oxidation rate at 34.77±6.18 unit/min. Combined treatment of curcumin(concentration ranged of 1-4 μg/mL) together with Hb(gly) or Hp1-1/Hb(gly) or Hp2-2/Hb(gly) showed a reduction in oxidation rate in a dose-dependent manner.

Table 13 Effect of curcumin treatment on oxidation rate in HepG2 induced Hb(gly) and Hp/Hb(gly) at concentration of 1 μg/mL. The data are presented as the mean ± standard deviation (SD).

Curcumin (μg/ml)	**Oxidation rate (unit/min)**		
	Hb	**Hp1-1/Hb(gly)**	**Hb**
0	32.72±5.80	30.69± 5.27	34.77±6.18
1	28.76±6.20	29.38± 5.83	32.35±4.94
2	27.88±6.28	27.91± 6.32	28.92±4.89
4	25.62±6.34	25.34± 4.68	26.83± 3.61
L1 1 mM	5.67±1.05	5.58± 0.45	5.93± 0.94

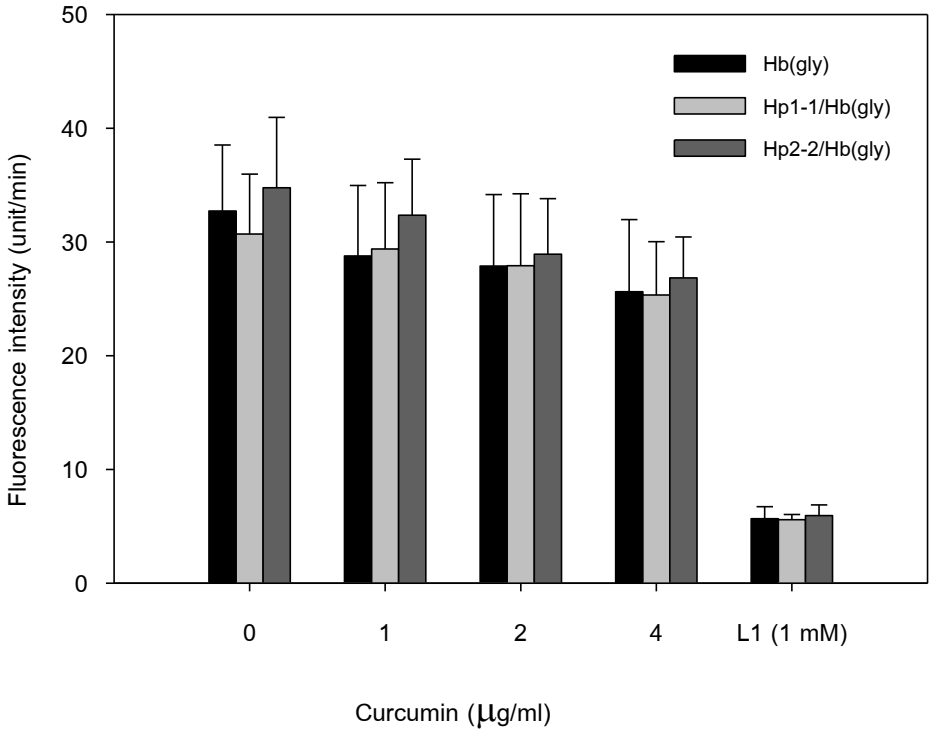


Figure 14 Effect of curcumin treatment on oxidation rate in HepG2 induced Hb(gly) and Hp/Hb(gly) at concentration of 1 μg/mL. The data are presented as the mean ± standard deviation (SD).

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