EFFECTS OF CYTOKINES ON NITRIC OXIDE MEDIATED ERYTHROID PROGENITOR CELLS APOPTOSIS IN β-THALASSEMIA/HEMOGLOBIN E AND ANEMIA OF CHRONIC DISEASE

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ABSTRACT

Anemia in β-thalassemia/hemoglobin E and rheumatoid arthritis anemia from chronic diseases are cause by different pathology. Ineffective erythropoiesis and apoptosis are the major causes of anemia. However, there has been incomplete information on the mechanism. The purpose of this study was to investigate the mechanism underlying the pathogenesis of these diseases, focusing on cytokine-induced nitric oxide production mediated by erythroid progenitor cell apoptosis of β-thalassemia/HbE and anemia from chronic disease. In this study, erythroid progenitor cells from healthy subjects, β-thalassemia/HbE and anemia from chronic diseases were cultured with cytokines, interleukin-1β, tumor necrosis factor-α, and interferon-γ at 2, 20 and 40 ng/ml concentration for 14 days. The effect of cytokines on total cell count and cell viability were then studied by trypan blue staining, and cell apoptosis was analyzed by flow cytometry. Moreover, the effect of cytokine on nitric oxide mediated cell apoptosis was examined by measuring nitric oxide production by Griess method and iNOS mRNA expression by Real-time PCR, respectively. The results showed that cytokines increased apoptosis of erythroid progenitor cells as determined by reduction of cell count, and cell viability, and induction of cell apoptosis. In addition, increased amounts of nitric oxide production and iNOS mRNA expression were found in erythroid cells cultured with cytokine, especially 20 ng/ml IFN-γ. Finally, percent apoptosis was reduced after an inducible nitric oxide synthase inhibitor, SMT treatment. It is the conclusion that cytokines could induce apoptosis in erythroid progenitor cells, and nitric oxide might be mediated in an apoptotic signaling pathway, which could play a role in pathogenesis of β-thalassemia/HbE and anemia due to chronic disease.

KEY WORDS: β-THALASSEMIA/HEMOGLOBIN E/ APOPTOSIS/ CYTOKINES/ NITRIC OXIDE

124 pages

ผลของไซโตคายน์ต่อการสร้างไนตริกออกไซด์ที่ทำให้เกิดการตายแบบอะพอพโตซิสในเซลล์เม็ดเลือดแดงตัวอ่อนของผู้ป่วยเบต้าธาลัสซีเมีย/ฮีโมโกลบินอี และภาวะโลหิตจางในโรคเรื้อรัง

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บทคัดย่อ

โลหิตจางในเบต้าธาลัสซีเมีย/ฮีโมโกลบินอีและภาวะโลหิตจางในโรคเรื้อรังโดยเฉพาะโรคข้ออักเสบรูมาตอยด์มีกลไกการดำเนินโรคที่แตกต่างกัน การสร้างเม็ดเลือดแดงบกพร่องและการตายของเซลล์เม็ดเลือดแดงแบบอะพอพโตซิสเป็นสาเหตุใหญ่ของภาวะโลหิตจาง อย่างไรก็ตามยังไม่มีข้อมูลที่ชัดเจนเกี่ยวกับกลไกดังกล่าว จุดประสงค์ของการวิจัยครั้งนี้ต้องการศึกษาผลของไซโตคายน์ต่อการสร้างไนตริกออกไซด์ที่ทำให้เกิดการตายแบบอะพอพโตซิสในเซลล์เม็ดเลือดแดงตัวอ่อนของผู้ป่วยเบต้าธาลัสซีเมีย/ฮีโมโกลบินอีและภาวะโลหิตจางในโรคเรื้อรังเปรียบเทียบกับคนปกติ โดยทำการเพาะเลี้ยงเซลล์เม็ดเลือดแดงตัวอ่อนจากผู้ป่วยเบต้าธาลัสซีเมีย/ฮีโมโกลบินอี, ภาวะโลหิตจางในโรคเรื้อรัง และคนปกติกับไซโตคายน์ชนิด interleukin-1β, tumor necrosis factor-α, และinterferon-γ ที่ความเข้มข้น 2, 20 และ40 ng/ml เป็นเวลา 14 วันแล้วศึกษาผลของ จำนวนเซลล์ทั้งหมด, จำนวนเซลล์ที่มีชีวิตอยู่โดยวิธีย้อมสี trypan blue และการตายของเซลล์แบบอะพอพโตซิสโดยวิธีโฟลไซโตเมทรี นอกจากนี้ยังทำการศึกษาบทบาทของไนตริกออกไซด์ต่อการกระตุ้นให้เกิดการตายแบบอะพอพโตซิสของเซลล์ โดยทำการศึกษาปริมาณของไนตริกออกไซด์โดยวิธี Griess assay และการแสดงออกของ iNOS mRNA โดยวิธี Real-time PCR ผลการศึกษาพบว่าไซโตคายน์สามารถทำให้เกิดการตายแบบอะพอพโตซิสเพิ่มขึ้นในเซลล์เม็ดเลือดแดงตัวอ่อนของผู้ป่วยเบต้าธาลัสซีเมีย/ฮีโมโกลบินอี, ภาวะโลหิตจางในโรคเรื้อรังและคนปกติตามลำดับ โดยเซลล์ที่เพาะเลี้ยงด้วยไซโตคายน์โดยเฉพาะ 20 ng/ml IFN-γ มีจำนวนเซลล์ทั้งหมดและจำนวนเซลล์ที่มีชีวิตอยู่ลดลง และพบการตายของเซลล์แบบอะพอพโตซิสมากที่สุด นอกจากนี้เซลล์ที่ถูกกระตุ้นให้เกิดการตายแบบอะพอพโตซิสโดยไซโตคายน์มีปริมาณของไนตริกออกไซด์และการแสดงออกของยีน iNOS มากกว่ากลุ่มที่ไม่เติมไซโตคายน์ เมื่อทดสอบด้วยการใช้ iNOS inhibitor พบว่าสามารถยับยั้งการสร้างไนตริกออกไซด์และการตายของเซลล์แบบอะพอพโตซิสได้ จากผลการศึกษาสามารถสรุปว่าไซโตคายน์ทำให้เซลล์เม็ดเลือดแดงตัวอ่อนตายแบบอะพอพโตซิสได้ และไนตริกออกไซด์น่าจะมีบทบาทสำคัญในขบวนการตายแบบอะพอพโตซิสในเซลล์เม็ดเลือดแดงตัวอ่อนของผู้ป่วยเบต้าธาลัสซีเมีย/ฮีโมโกลบินอีและภาวะโลหิตจางในโรคเรื้อรัง

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LIST OF ABBREVIATIONS

Abbreviations	Terms
ACD	Anemia of chronic disease
ACR	American College of Rheumatology
Bp	Base pair
CaM	Calmodulin
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CO_2	Carbondioxide
DNA	Deoxyribonucleic acid
DW	Distril water
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
FADD	Fas associated death domain
FBS	Fetal bovine serum
FMN	Flavin mononucleotide
g/dl	Gram per desiliter
Hb	Hemoglobin
Hct	Hematocrit
IL	Interleukin
IFN	Interferon
IMDM	Iscove's modified Dulbecco's medium
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
Min	Minute

LIST OF ABBREVIATIONS (cont.)

Abbreviations	Terms
ml	Mililiter
mM	Millimole
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa B
ng	Nanogram
nm	Nanomole
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drug
O.D	Optical Density
Pg	Picogram
PLT	Platelet
PS	Phosphatidylserine
RA	Rheumatoid arthritis
RBC	Red blood cell
RDW	Red cell distribution width
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Revolution per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard Deviation
Sec	Second
SMT	S-Methylisothiourea Sulfate
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
TIBC	Total iron binding capacity

LIST OF ABBREVIATIONS (cont.)

Abbreviations	Terms
TNF-α	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
WBC	White blood cell

CHAPTER I
INTRODUCTION

Oxidative stress is an imbalance between the production of reactive oxygen species and the antioxidant system that functions to detoxify the reactive intermediate. In human, oxidative stress is involved in several pathological conditions and diseases. Many causes of oxidative stress come from hydroxyl radicals, superoxide anion, hydrogen peroxide and nitric oxide (1). Nitric oxide ($^{\cdot}$NO) is a very small and short-lived free radical molecule that diffuses freely within the cell. In normal condition, $^{\cdot}$NO acts as a biological mediator similar to neurotransmitters in the neuronal system regulate blood vessel in vascular systems, and is an important host defence effector in the immune system. On the other hand, it is a free radical and acts as a cytotoxic agent in pathological diseases, particularly in inflammatory disorders. The production of $^{\cdot}$NO in the body is catalyzed by a family of enzymes called nitric oxide synthases (NOSs). Currently, there are three isoforms of NOS: endothelial NOS or eNOS, neuronal NOS or nNOS and inducible NOS or iNOS. Inducible NOS (iNOS), is not present in resting cells but can be induced by immunostimulatory cytokines include TNF-α, IL-1β and IFN-γ, bacterial products or infection in a number of cells, including endothelium, hepatocytes, monocytes, mast cells, macrophages and smooth muscle cells (2). The research suggest that cytokines may involved toxic effects on the cells by inducing the production of $^{\cdot}$NO in pathological of the diseases (3).

Thalassemias are heterogeneous group of inherited anemia resulting from reduced or absent synthesis of alpha or beta globin chains of hemoglobin (4), which carries oxygen to all parts of the body. The clinical severity varies, ranging from asymptomatic to severe or even fatal entities. The incidence of α thalassemia is 30% to 40% in Northern Thailand and Laos, and it is usually divided into α^0 thalassemia (--/αα) and α^+ thalassemia (-α/αα) according to the number of α-globin genes that are either deleted or nonactive. The α^0 thalassemia prevalence in Thailand between 3.6%

and 10%, whereas α^+ thalassemia is much more frequent (16.4 % to 20 %). β-thalassemia occurs with less frequency, about 9 % in Thailand. HbE is common in Thailand with 13% prevalence on the average. These disorders lead to different combinations over 60 different thalassemic syndromes. Approximately 5.6% of Thai couples are at risk of giving birth babies with severe hemoglobinopathies, such as Hb Bart's hydrops fetalis, β-thalassemia major, and β-thalassemia/HbE (5).

β-thalassemia/HbE is characterized by the absence or reduced synthesis of beta globin chains. The presence of hemoglobin E and F, with the amount of hemoglobin E ranging from 35% to 75% (6). Anemia in β-thalassemia/HbE is caused by a combination of ineffective erythropoiesis and premature hemolysis of red blood cells in peripheral circulation. An excess pool of unpaired alpha hemoglobin, which precipitate in the red blood cell precursors and excess unbound iron, leading to increased susceptibility of thalassemic red blood cells (RBC) to oxidative stress (6). Furthermore, β-thalassemic patients are under continuous blood transfusion leading to iron overload. Iron is known to be a catalyst in the formation of reactive oxygen species (ROS) (6, 7). Moreover, microenvironments such as cytokine or growth factor might be effected in erythroid progenitor cells apoptosis because recent research showed thalassemic patients had significantly increase level of IL-1β, TNF-α and IFN-γ (8).

Anemia of chronic disease (ACD) is the most frequent among hospitalized patients develops under chronic inflammatory disorder such as chronic infections, cancer or autoimmune diseases (9). In chronic inflammation, activated monocyte and T lymphocyte produce inflammatory cytokines including TNF-α, IL-1β, IFN-γ and IL-4. Pathogenesis of the anemia is still unclear but there are many studies focus on three areas includes dysregulation iron homeostasis, impaired erythropoiesis and blunted erythropoietin response (10). It has been reported that ACD in rheumatoid arthritis shown increase proinflammatory cytokine such as TNF-α and IFN-γ. In addition, several study undertaken on patients with RA and SLE have documented increase NO synthesis (11). The evidence showed increased proinflammatory cytokines such as IL-β and TNF-α can induce iNOS expression in many cells (12), that may be involved in pathology of erythroid progenitor cell. Apoptosis of β-thalassemia/HbE and rheumatoid arthritis patients have not been investigated. Therefore, the goal of this

study is to investigate the mechanism underlying the pathogenesis of β-thalassemia/hemoglobin E and anemia of chronic disease focusing on cytokines induced $^{\cdot}NO$ production involves in erythroid progenitor cells apoptosis. The information from this study could be benefit for better understanding of pathogenesis of both diseases.

CHAPTER II
OBJECTIVES

Anemia in β-thalassmia/hemoglobin E and anemia of chronic disease may cause from difference pathology. Knowledge of the mechanisms of erythroid progenitor cells apoptosis would provide a clue to understand pathology of the diseases. However, the mechanism and regulation of cytokine induced apoptosis in erythroid progenitor cells still have not been completely investigated.

The aims of this thesis are

1. Study the effect of cytokines, Interleukin 1 beta (IL-1β), Tumor necrosis factor alpha (TNF-α) and Interferon gamma (IFN-γ) on cell viability and cell apoptosis of erythroid progenitor cells from β- thalassemia/hemoglobin E and anemia of chronic diseases.
2. Study the effect of cytokines, Interleukin 1 beta (IL-1β), Tumor necrosis factor alpha (TNF-α) and Interferon gamma (IFN-γ) on inducible nitric oxide synthase expression and nitric oxide production involved in apoptosis of β-thalassemia/hemoglobin E and anemia of chronic disease.

CHAPTER III
LITERATURE REVIEW

3.1 Erythropoiesis

Hematopoiesis is the process through which the organism acts to renew the cells and formed elements of blood. This complex and vital process represents the result of many interactions among various growth factors and progenitor cells which give rise to the mature cells of the hematopoietic system. Erythropoiesis is a subset of this larger scheme, including only the events that lead from the appearance of the committed erythroid progenitor cell through the formation of mature red blood cells (13). Erythropoiesis is the development of mature red blood cells (erythrocytes). Like all blood cells, erythroid cells begin as pluripotential stem cells. The first cell that is recognizable as specifically leading down the red cell pathway is the proerythroblast. As development progresses, the nucleus becomes smaller and the cytoplasm becomes more basophilic, due to the presence of ribosomes. In this stage the cell is called a basophilic erythroblast. The cell will continue to become smaller throughout development. As the cell begins to produce hemoglobin, the cytoplasm attracts both basic and eosin stains, and is called a polychromatophilic erythroblast. The cytoplasm eventually becomes more eosinophilic, and the cell is called an orthochromatic erythroblast. This orthochromatic erythroblast will then extrude its nucleus and enter the circulation as a reticulocyte. Reticulocytes are so named because these cells contain reticular networks of polyribosomes. As reticulocytes loose their polyribosomes they become mature red blood cells (14-15).

A feedback loop involving erythropoietin helps regulate the process of erythropoiesis so that, in non-disease states, the production of red blood cells is equal to the destruction of red blood cells and the red blood cell number is sufficient to sustain adequate tissue oxygen levels but not so high as to cause sludging, thrombosis, or stroke. Erythropoietin is produced in the kidney and liver in response to low oxygen levels. In addition, erythropoietin is bound by circulating red blood cells, low

circulating numbers lead to a relatively high level of unbound erythropoietin, which stimulates production in the bone marrow (16).

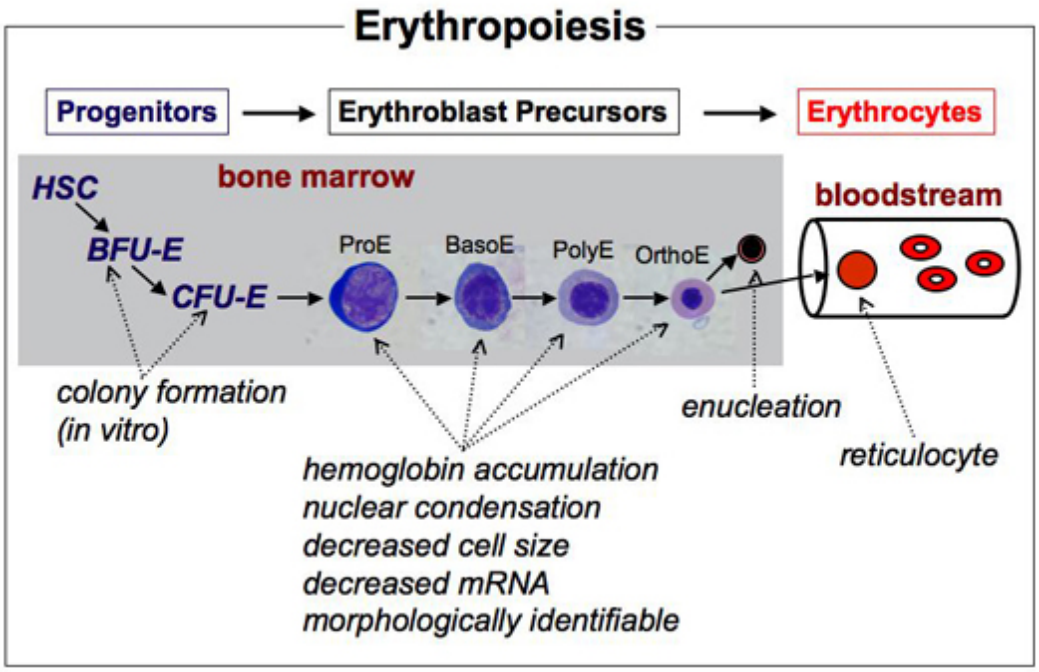


Figure 1. Erythropoiesis processes (17).

3.2 Thalassemia

Thalassemias are a group of inherited autosomal recessive hematologic disorders that cause hemolytic anemia because of the decreased or absent synthesis of a globin chain. The decreased production of one type of globin leads to imbalance of globin chains cause hemolysis and impair erythropoiesis. The clinical outcome from this genetic mutation is extremely wide ranging from no anemia to fetal death. Usually, the synthesis of either the alpha (α) or beta (β) chains is impaired, the thalassemias are named according to the chain with reduced or absent synthesis: alpha (α) thalassemia, which is caused by a defect in the rate of synthesis of α chain, and beta (β) thalassemia, caused by a defect in the rate of synthesis of β chains (4).

Hemoglobin consists of an iron containing heme ring and four globin chains: two alpha and two non-alpha. The composition of the four globin chains determines the hemoglobin type. Fetal hemoglobin (HbF) has two alpha and two gamma chains ($\alpha_2\gamma_2$). Adult hemoglobin A (HbA) has two alpha and two beta chains

($\alpha_2\beta_2$), whereas hemoglobin A_2 (HbA_2) has two alpha and two delta chains ($\alpha_2\delta_2$). At birth, HbF accounts for approximately 80 percent of hemoglobin and HbA accounts for 20 percent. The transition from gamma globin synthesis (HbF) to beta globin synthesis (HbA) begins before birth. By approximately six months of age, healthy infants will have transitioned to mostly HbA, a small amount of HbA_2, and negligible HbF (Figure 2) (17-18).

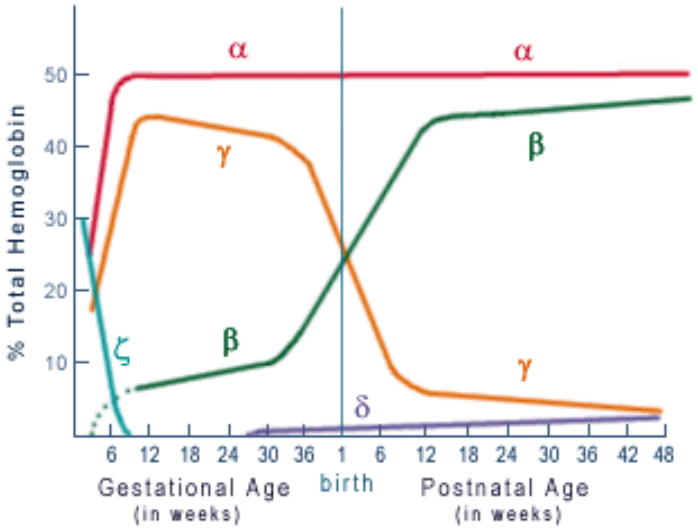


Figure 2. The synthesis of the globin chain (19)

Although approximately 7% of the world's population have a thalassemic condition, the distribution is concentrated in the "thalassemia belt", which extends from the Mediterranean East though the Middle East and India to Southeast Asia and South Africa (20-21). The incidence of β-thalassemia ranges from 1% to 20% depending on the region, in Sardinia, Crypus, and Greece having the highest numbers in Europe (6% to 19%), while Thailand and Cambodia having the highest number of Southeast Asia (1% to 11%) (21). The incidence of α-thalassemia varies considerably. In Europe, Sardinia has the highest carrier frequency (12%), whereras in tribe in India and Papua New Guinea the frequency is 80% (Figure 3) (21).

In Thailand, thalassemias have high incidence rate and trend to increase everyear. The gene frequencies of α-thalassemia reach 20-30%, β-thalassemia vary between 3-9% and 20-30% of which having a co-incidence with hemoglobin (Figure

4) (22-23). More than 20 million people are affected with thalassemia carrier leading to the birth of new cases with thalassemia disease approximately 12,125 cases per year. Approximately 50% of these new cases have the severe thalassemia including Hb Bart's hydrops fetalis, homozygous β-thalassemia and β-thalassemia/HbE diseases. So, an effort has been made by both the government and non government organization to provide better care for thalassemics as well as to promote prevention and control (23).

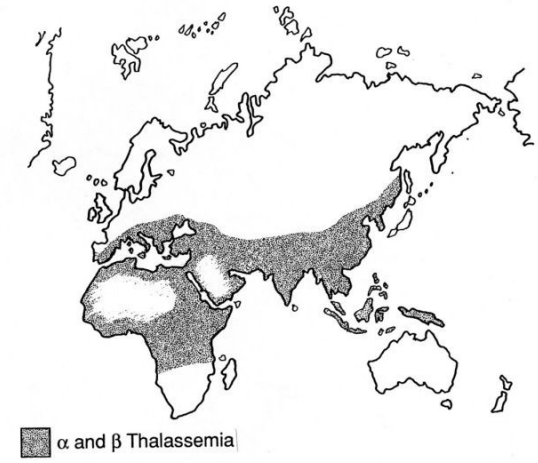


Figure 3. The geographical distribution of the thalassemias (21)

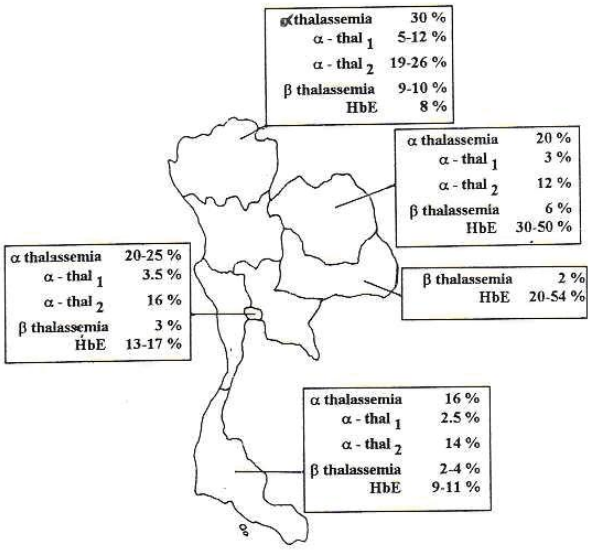


Figure 4. The incidence of thalassemias in Thailand (23).

3.2.1 Alpha thalassemia

The α-thalassemias show several important differences from β-thalassemia. Because α chains are shared by fetal and adult hemoglobin, the disease is manifest in both fetal and adult life. Furthermore, excess γ and β chains do not precipitate immediately in the bone marrow like α chains but produce the physiologically useless and unstable tetramers: γ_4 (hemoglobin Bart's) and β_4 (hemoglobin H). Since the α genes are duplicated the genetics of α-thalassemia is more complicated than β-thalassemia. The genetic of normal individuals can be written αα/αα. Loss of both α genes on a chromosome is called α° thalassemia, and is represented --/αα. Loss of one of the linked pairs of a globin genes is called α^+ thalassemia, -α/αα. Usually these genes are lost by deletion, though sometimes they are inactivated by a point mutation, as is the case in the β-thalassemia (24).

The homozygous state for α° thalassemia produces intrauterine death with a profoundly anemia as the hemoglobin Bart's hydrops fetalis syndrome. Mothers carrying babies of this type commonly have toxemia of pregnancy and postpartum bleeding. Compound heterozygotes for α° and α^+ thalassemia, --/-α, have a milder illness characterized by anemia and splenomegaly which is called hemoglobin H disease. Carriers for α° thalassemia (--/αα) and homozygotes for α-thalassemia (-α/-α) have a mild hypochromic anemia, while carriers for α^+ thalassemia have no hematological abnormalities (24).

3.2.2 Beta thalassemia

This results in imbalanced globin chain synthesis and production of an excess unpaired α chains, which precipitate in the red blood cell precursors and excess unbound iron, leading to increased susceptibility of β-thalassemic red blood cells to oxidative stress, that damage red cell membranes, resulting in intravascular hemolysis. Premature destruction of erythroid precursors results in intramedullary death and ineffective erythropoiesis, an abnormal and high rate of premature erythroblast production. This process causes severe anemia, which in turn leads to increased erythropoietin production and expansion of the ineffective bone marrow, bone deformities, splenomegaly and growth retardation. The severity of β-thalassemia depends in part on the type of β-thalassemic genes that a person has inherited (25).

β-thalassemia heterozygote has one β globin gene that is normal, and an affected gene with a variably reduced production of β globin. The degree of imbalance with the α globin depends on the residual production capacity of the defective β globin gene. Even when the affected gene produces no β chain, the condition is mild since one β gene functions normally. The red cells are small and a mild anemia may exist. People with the condition generally have no symptoms. The condition can be detected by a routine laboratory blood evaluation. In many ways, the one gene β-thalassemia and two gene α-thalassemia are very similar, from a clinical point of view. Both results are small red cells and a slightly mild anemia.

β-thalassemia homozygote produces a severe anemia and a potentially life-threatening condition. The severity of the disorder depends in part on the combination of genes that have been inherited: β^0/β^0-thal, β^0/β^+-thal, β^+/β^+-thal. The β^+-thalassemia genes vary greatly in their ability to produce normal hemoglobin. Consequently, the clinical picture is more complex than heterozygote might otherwise be the case for three genetic possibilities outlined (26).

3.3 Beta-thalassemia/Hemoglobin E

Worldwide, β-thalassemia/HbE may be the most important hemoglobinopathy because of the high gene frequencies for both HbE and β-thalassemia. Since the classic description of β-thalassemia/HbE by Chernoff, it has been noted to be an important hemoglobin disorder in the Indian subcontinent and Southeast Asia (27-29). The hemoglobin concentrations ranged from 3 to 13 g/dL with an average of 7.7 g/dL (30-31). The compound heterozygote state of β-thalassemia/HbE results in a variable, often severe anemia, with the phenotype ranging from a complete lack of symptoms to transfusion dependence (32). Approximately one-half of the patients are phenotypically similar to patients with thalassemia major who require regular transfusion therapy. The clinical course for the other half resembles thalassemia intermedia. The classification of thalassemia intermedia includes a diverse group of patients. As some of the patients with thalassemia grow older, they become transfusion dependent. Others remain asymptomatic throughout late adulthood (33). Even with screening for genetic modifiers, early predictors of

severity have yet to be defined. The time of onset and the severity of anemia may be useful in predicting the long term clinical phenotype. Splenomegaly often develops in severely affected patients. In the past, splenectomy was routinely performed in an attempt to increase hemoglobin levels. The bone marrow expansion and increased metabolic rate that are found in β-thalassemia/HbE result in growth failure, delay in secondary sex development and osteoporosis. In older patients, severe bone pain may lead to the initiation of transfusion therapy. Iron overload in nontransfused patients is common, secondary to increased gastrointestinal absorption of iron.

The pathophysiology of β-thalassemia/HbE are complex. Ineffective erythropoiesis, apoptosis, and oxidative damage are central components of the disease and its shortened red blood cell survival (34-36). Its characterized by the absence or reduced synthesis of β-globin chain lead to unbalance globin chain. Synthesis accumulation of unpaired α-globin in red blood cell precursors are unsoluble and precipitate in the cell lead to destruction of red blood cell precursors and ineffective erythropoiesis. Surviving cells that arrived peripheral blood with intracellular inclusion bodies are sample to hemolysis (Figure 5). Oxidative damage with deficiency of antioxidants may also be important in the cellular pathophysiology of this disease (37). Futhermore, possibility cause of apoptosis may associate with microenvironment in the cells such as cytokines included TNF-α, IL-1β. In recent studies, β-thalassemia/HbE patients had significantly increase level of IL-1β, TNF-α and IFN-γ, which may involve in erythroid progenitor cell apoptosis by induce nitric oxide production in the cells.

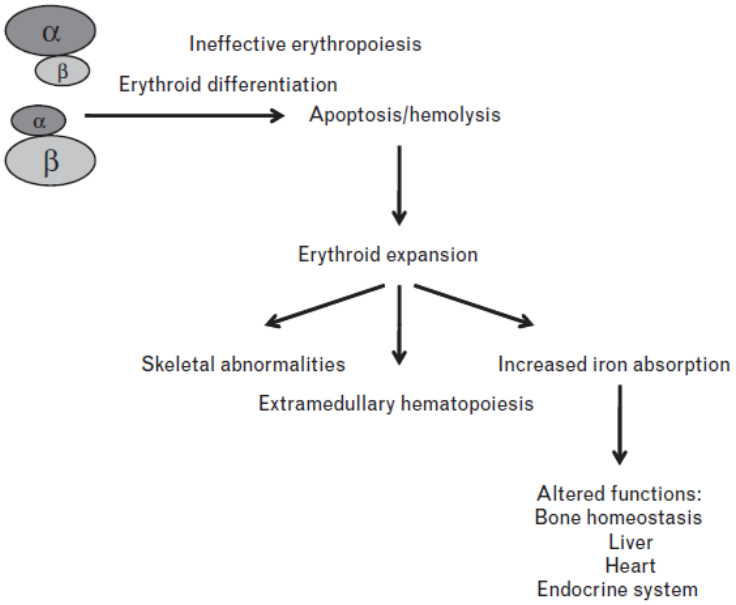


Figure 5. Pathophysiology of β-thalassemia/hemoglobin E (35)

3.4 Anemia of chronic disease

Anemia of chronic disease (ACD), the most frequent anemia among hospitalized patients, develops under chronic inflammatory disorders such as chronic infections, cancer or autoimmune diseases (9). Anemia of chronic disease (ACD) is a mild to moderate normocytic-normochromic or, less frequently, microcytic-hypochromic anemia characterized by decreased serum iron, decreased total iron-binding capacity and increased iron stores. Reticulocytes levels are normal to reduced and classify ACD among hyporegenerative anemias. Although ACD is a frequent and easily diagnosed clinical entity, its pathogenesis still remain unclear. The high incidence and the heterogeneity of chronic disorders that may present ACD have two implications. First, ACD is one of the most common forms of anemia. Second, differences are to be expected in the pathogenesis of and potential therapeutic approaches to ACD occurring in different clinical settings. Systemic autoimmune diseases are frequently characterized by ACD, and may benefit from a more unified immunological and hematological definition. Therefore, it can be useful to review a number of informations that relate erythropoiesis to immune hyperactivity. More specifically, there are three areas of major interest. First, the organism's reaction to

inflammatory stimuli and the effect of proinflammatory cytokines on erythropoiesis. Second, the production and activity of erythropoietin (EPO). Third, the possibility that cell-mediated immunity may influence iron metabolism through its effects on NO synthesis (38).

Table 1. Cause of anemia of chronic diseases (9).

Associated diseases	Estimated prevalence (%)
Infections (acute and chronic)	18-95
- Viral	
- Bacteria	
- Parasitic	
- Fungal	
Cancer	30-77
- Hematologic	
- Solid tumor	
Autoimmune	8-71
- Rheumatoid arthritis	
- SLE	
- Vasculitis	
Chronic rejection after solid-organ transplantation	8-70
Chronic kidney disease and inflammatory	23-50

3.4.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by symmetric chronic inflammation of small and large joints. Destruction of cartilage and periarticular bone results from the release of neutral proteases and matrix metalloproteinases as part of the inflammatory process within the synovium. Although RA originates in the synovial tissues of the joints, it is a systemic disease that in its

more severe form may include rapidly progressive multisystem inflammation. The most common symptoms of RA manifested in the joints are inflammation and swelling, which are usually accompanied by pain, discomfort, and periods of prolonged morning stiffness. Additional common symptoms that are more systemic in nature include loss of muscle strength and low general energy levels. Any and all of these symptoms are likely to have a negative impact on the quality of life of patients with RA (39). RA has been estimated to occur in approximately 1% of the adult US population, there are approximately 30,000 to 50,000 prevalent (active and inactive) cases of juvenile RA (40-41). The etiology of the disease appears to be multifactorial with genetic, environmental, and endocrinologic factors all playing roles (42-43).

Anemia is a common extra-articular manifestation in patients with rheumatoid arthritis (RA). The exact mechanisms underlying anemia of chronic disease (ACD) in RA is unknown but is possibly related to inflammatory cytokines mediated pathogenesis, which include defective production of erythropoietin, reduced bone marrow response to erythropoietin, defective reticulo-endothelial release of iron causing iron deficit erythroblast. The estimated prevalence of anemia in RA ranges from 33.3 to 59.1%. The difference in prevalence rate in various studies is related to the difference in the definition of anemia. The reported prevalence of anemia defined as WHO criteria in adult Thailand population is 47.5%. Its prevalence is higher in women than in men. Anemia adds considerably to the morbidity in patients with RA. Improvement in hemoglobin levels is associated with significant improvement in quality of life of anemic patients with RA. ACD and iron-deficiency anemia (IDA) are the two most common causes of anemia in RA. IDA is the commonest cause of anemia worldwide. Iron deficiency in RA is mainly related to non-steroidal anti-inflammatory drugs (NSAID) induced chronic gastrointestinal blood loss. Poor intake as well as other dietary factors may contribute to iron deficiency in RA patient. Anemia of chronic disease is often closely resembles iron-deficiency anemia. Indeed, many people with chronic disease can also be iron deficient, and the combination of the two causes of anemia can produce a more severe anemia. Differentiation between iron deficiency anemia (IDA) and anemia of chronic disease (ACD) are in the overall evaluation of iron status.

The American College of Rheumatology (ACR) has defined (1987) the following criteria for the classification of rheumatoid arthritis include, morning stiffness of >1 hour most mornings for at least 6 weeks, arthritis and soft-tissue swelling of >3 of 14 joints/joint groups, present for at least 6 weeks, arthritis of hand joints, present for at least 6 weeks, symmetric arthritis, present for at least 6 weeks, subcutaneous nodules in specific places, rheumatoid factor at a level above the 95th percentile, and radiological changes suggestive of joint erosion.

At least four criteria have to be met for classification as RA. These criteria are not intended for the diagnosis for routine clinical care; they were primarily intended to categorize research. For example: one of the criteria is the presence of bone erosion on X-Ray. Prevention of bone erosion is one of the main aims of treatment because it is generally irreversible. To wait until all of the ACR criteria for rheumatoid arthritis are met may sometimes result in a worse outcome. Most sufferers and rheumatologists would agree that it would be better to treat the condition as early as possible and prevent bone erosion from occurring, even if this means treating people who don't fulfill the ACR criteria. The ACR criteria are, however, very useful for categorizing established rheumatoid arthritis, for example for epidemiological purposes (44-45).

RA is associated with increased production of $^{\cdot}$NO, due to activation of iNOS pathway. Studies in animal models have suggested that $^{\cdot}$NO plays a causal role in the pathogenesis of joint inflammation and tissue damage, since the severity of arthritis can be reduced by the administration of NOS inhibitors. Several cell types present within the joint, including synovial fibroblasts, endothelial cells and chondrocytes, can be induced proinflammatory cytokines to produce $^{\cdot}$NO *in vitro* (46-48).

3.5 Cytokines

Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They generally act over short distances and short time spans and at very low concentration. They act by binding to specific membrane receptors, which then signal the cell, like hormones and neurotransmitters,

which are used for inter-cell communication. Cytokines are produced by a many types of cell in hematopoietic and non-hematopoietic and can effect on nearby cells or throughout the organisms (49). They include the families of interleukins, interferons, colony-stimulating factors, IL-6 family, and chemokines (Table 2). While each cytokine has unique and specialized roles, the majorities of cytokines are pleiotropic in their function activities and are extracellular signaling molecules essential to host development and maturation. Immune, inflammatory response and tissue repair processes (50). Moreover, cytokines can regulate proliferation and differentiation in various hematopoietic components. For example, IL-2 promotes proliferation and differentiation of B cell and activates macrophages, IL-3 stimulates the proliferation of precursors in all hematopoietic lineages (51). However, some cytokines also are negative regulators. Such as TNF-α suppresses erythropoiesis (52). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induced both cytotoxic (apoptosis) and cytostatic effects in the human myeloid K562 cell line (53). Interferon-γ, a lymphokine produced by activated T cells, is an important regulatory molecule of the immune system. IFN-γ has been implicated in the pathogenesis of multiple sclerosis, diabetes mellitus, and other human autoimmune diseases. Whereas most investigators have reported negative effects of IFN-γ on hematopoiesis in vitro, in some laboratories this cytokine has either not been inhibitory of hematopoietic colony culture conditionally suppressive or even stimulatory, depending on the choice of culture conditions and target cells.

Moreover, there are recent studies shows that cytokines can also stimulate the production of nitric oxide by upregulate iNOS expression. For instance, interleukin-1β (IL-1β) induces expression of the inducible nitric oxide synthase (iNOS) with concomitant release of nitric oxide ($^{\cdot}$NO) from glomerular mesangial cells (12) and cardiac fibroblast cell (54).

Table 2. Cytokine families consist of interleukins, interferons, colony-stimulating factor, IL-6 Family, TNF Family and Chemokines.

Interleukins	IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18
Interferons	IFN-α, IFN-β, IFN-γ, IFN-Ω
Colony-stimulating factors	G-CSF, GM-CSF, M-CSF, EPO, IL-3, SCF
IL-6 Family	IL-6, IL-11, OSM, LIF
TNF Family	TNF-α, TNF-β, FasL, CD40L, TRAIL, APRIL
Chemokines	Lymphotactin, MCP-1, MCP-2, MCP-3, MCP-4, MIP-1s, MIP-3, MIP-4, MIP-5, IL-18

3.5.1 The IL-1 family

Interleukin-1 (IL-1) is one of the most important cytokine in immune response. The predominant function of IL-1 is to enhance the activation of T-cells in response to antigen. The IL-1 are secreted primarity by macrophages but also from neutrophils, endothelial cells, smooth muscle cells, B and T cells, fibroblasts and keratinocytes. Production of IL-1 by these different cell types occurs only in response to cellular stimulation (55). This cytokine is involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis (56). The IL-1 family composes of three ligands: IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra). IL-1α and IL-1β are synthesized by multiple cells including monocytes, macrophages, neutrophils, hepatocytes and tissue macrophages throughout the body. In the human, pro IL-1α is synthesized in the cytoplasm as a 31kDa precursor that is biologicallyactive, is capable of binding to type IL-1R and activating cells. Pro-IL-1α may be cleaved by calpain, a membrane bound cysteine protease that require calcium, leading to release of mature 17 kDa IL-1α from the cell. However, the majority of IL-1α remains either bound to the plasma membrane or inside the cell with some localization in the nucleus. Pro IL-1α may travel to the nucleus and serve as an autocrine growth factor. IL-1β is also synthesized in the cytoplasm as a 31 kDa molecule and is processed and releases from cells by a mechanism involving an

enzyme called IL-1β-converting enzyme (57), now known as caspase-1. Pro-1β is biologically inactive and must be converted to the mature 17 kDa IL-1β to acquire the ability to bind to receptors and activate cells. Although, IL-1β is the predominant in humans while it is IL-1α in mice (55).

There are two forms of interleukin-1 receptor (IL-1R). IL-1RI processes a long cytoplasmic domain and is capable of activating cells. IL-1RII has only a short intracellular domain and is biologically inert. However, IL-1RII may negatively regulate cell activation by acting in the membrane to compete for ligand binding with IL-1RI. In addition, IL-1RII is readily released from cells where it may bind IL-1 in the cell microenvironment, preventing interaction with cell surface IL-1RI. After IL-1 binds to IL-1RI, a second chain called the IL-1RAcP joins with IL-1/IL-1RI to form a complex, leading to cell activation mediated by the cytoplasmic domains of both receptor chain. This complex recruits a number of intracellular adapter molecules, including MyD88 (myeloid differentiation factor 88), IRAK (IL-1Rassociated kinase), and TRAF6 [tumor necrosis factor (TNF) receptor-associated factor 6], to activate signal transduction pathways such as nuclear factor-kB (NF-kB), AP-1 (activator protein-1), JNK (c-Jun N-terminal kinase), and p38 MAPK (mitogen-associted protein kinase).

Because each member of the IL-1 family binds to the same IL-1 receptors, it is not surprising that IL-1α, IL-1β and IL-1Ra share structure topology. Although the role of IL-1 on induction apoptosis has been report in the insulin-producing β cell of the pancreatic islets. The mechanism for this IL-1-induced death is via production of nitric oxide in cardiac myocyte (58). Although there are other examples of IL-1β induce apoptosis, in some cells IL-1 protects against cell death (55).

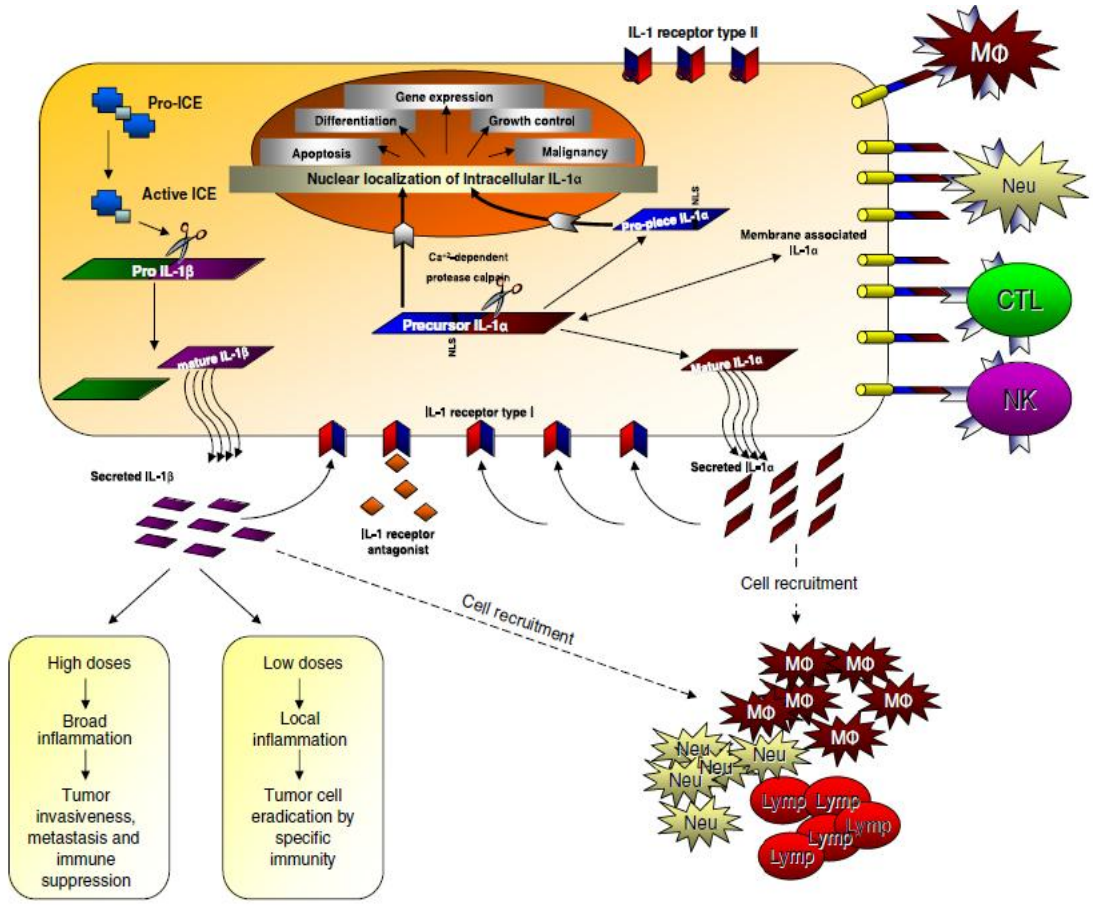


Figure 6. The models of action of the IL-1 molecules in cells and their possible influence on inflammatory and immune responses (56).

3.5.2 Tumor necrosis factor-alpha

The term tumor necrosis factor (TNF) refers to two closely related cytokines known as tumor necrosis factor alpha (TNF-α, cachectin) and tumor necrosis factor beta (lymphotoxin, TNF-β). Both cytokines interact with the same cell membrane receptors, and both have been implicated as pathogenic mediators of human illness. TNF is synthesized by macrophages and other cells in response to bacterial toxins, inflammatory products, and other invasive stimuli. The gene of human TNF encodes a prohormone that is inserted into the cell membrane as a polypeptide with an apparent molecular weight of 26 kDa. This membrane-bound form of TNF is bioactive as assayed by cell cytotoxicity and has been implicated in the paracrine activities of TNF in tissues. In response to bacterial endotoxin (lipopolysaccharide or LPS) and other stimuli, the 26 kDa form is proteolytically cleaved into a 17 kDa polypeptide

subunit composed of 157 amino acids. Three of these TNF monomers associate noncovalently to form a trimer. The resultant bell-shaped trimeric form is predominanting bioactive form of TNF in serum and other body fluids (59).

Two types of TNF receptors, type I (TNFR-55) and type II (TNFR-75), are present on the plasma membranes of virtually all cells. The receptors share structural homology in the extracellular TNF binding domains and exhibit similar bindind affinity for TNF, but they induce separate cytoplasmic signaling pathways following receptor ligand binding.

Tumor necrosis factor alpha (TNF-α) is a pleiotropic proinflammatory cytokine with a wide variety of functions in many cell types. TNF-α exerts its biological activity by binding to type 1 and type 2 receptors (TNF-RI and TNF-RII) and activating several signaling pathways. TNFRs belong to a large family of nerve growth factor receptors. These are type I transmembrane receptors with one to five cystein-rich repeats in their extracellular domains and a common death domain in their cytoplasmic tail (57). TNF-RI contains DD, whereas TNF-RII lacks DD. Therefore, TNF-RI signals both cell survival and cell death signals, whereas TNF-RII primirity mediates a cell survival signals (60).

Upon ligation with TNF-α, TNF-RI undergoes trimerization of its receptor. Because DDs do not posses any intrinsic enzymatic activity, they recruit an adapter protein, TNF-R associated death domain (TRADD), which in turn recruits another adapter molecule, the Fas-assoiated death domain (FADD). FADD recruits procaspase-8 by protein-protein interaction via homologous death effector domain (DED) to form a death inducing signal complex (DISC). During DISC formation procaspase-8 is autolytically cleaved to yield active caspase-8. Active caspase-8 is rapidly released from the DISC to cytoplasm and serves as an enzyme for downstream effector caspase-3. These effector caspases (especially caspase-3) cleaves a number of substrates resulting in morphologic and biochemical features of apoptosis. In certain cell types and under special conditions, caspase-8 may cleave Bid (BH3-interaction death domain agonist, a member of Bcl-2 family), which translocate to mitochondria cause release of cytochrome c and activation of caspase-9 and effector caspase-3 to induce apoptosis (61).

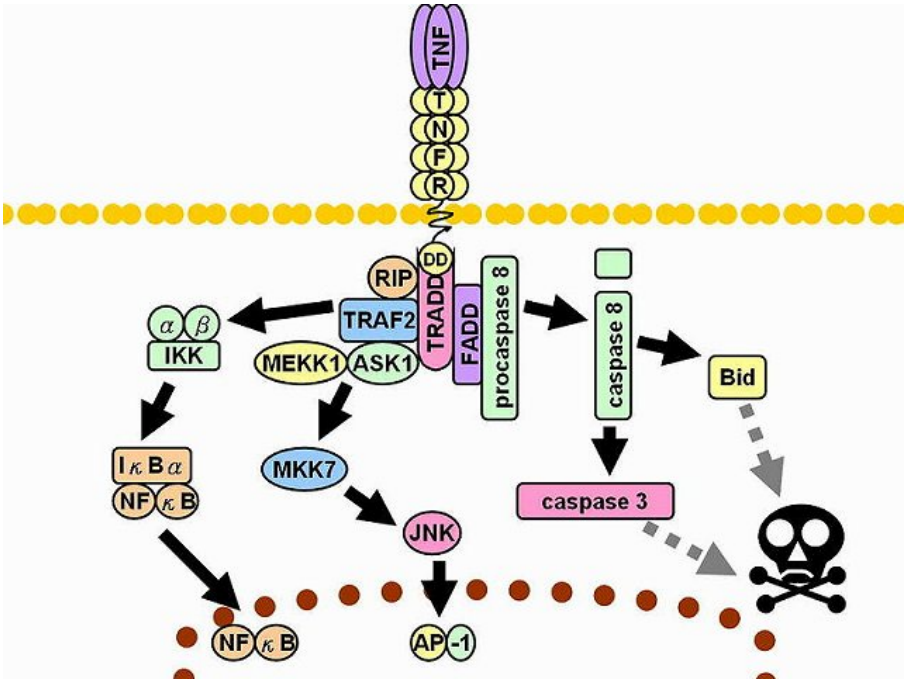


Figure 7. The models of action of the TNF molecules in cells and their mechanism (59).

3.5.3 Interferon

Interferons (IFNs) are a family of cytokines that elicit pleiotropic biological effects. Although identified and named for their ability to interfere with viral replication in treated cells, IFNs have immunomodulatory, cell differentiative, anti-angiogenic and anti-proliferative effects. For almost twenty years, IFN-α has been used to induce regression of human neoplasia. Chronic myelogenous leukemia, myeloma, hairy cell leukemia, Kaposi's sarcoma, lymphomas, melanomas, renal cell and bladder carcinomas have all responded to IFNs. Despite the beneficial effects of IFNs in some malignant diseases, even in relatively responsive conditions, a substantial proportion of patients fail to respond. Thus a better understanding of the mechanisms that underlie IFNs anti-tumor effect and the factors that are responsible for a lack of response to IFNs would undoubtedly lead to an improved use of IFN in malignant diseases.

IFNs consist of the multiple type I species (IFN-α, IFN-β, IFN-ω and IFN-κ), and the one type II species (IFN-γ). Type I IFNs, induced in most cell types by viruses and dsRNA, are clustered on chromosome 9 and consist of several α genes and

pseudogenes and one β gene. In contrast IFN-γ, mainly secreted by Th-1 lymphocytes and NK cells, is coded by a single gene on chromosome 12. Following secretion from cells, IFNs mediate their effects by binding to cell surface receptors and thus activating members of the JAK kinase family. Activated JAK kinase phosphorylate the signal transducers and activators of transcription (STAT) family of transcription factors. IFNs may elicit anti-tumor affects either by acting on tumor cells or on other cells. For example IFNs may affect the proliferation or induce cellular differentiation of tumor cells (62).

3.5.3.1 Interferon-gamma (IFN-γ)

Interferon (IFN-γ) acts in the defense of an organism foreign pathogens via cellular immunity mediated by macrophages. It is a product of T-helper 1 (Thl) cells that can modulate the function of Th2 lymphocytes. In general, Thl cytokines stimulate the body's cellular immunity in viral infections. IFN-γ is potentially antiviral, antimicrobial, anti-proliferative, anti-tumor, and anti-allergic. Many studies have revealed that IFN-γ plays a central role in iNOS expression, with subsequent synthesis of nitric oxide ($^{\cdot}$NO). Some results have shown that IFN-γ induced apoptosis could be responsible of iNOS in human hepatoma cell, moreover there are other families gene induced by IFN-γ. Some play a critical role in apoptosis, such as Fas, caspases, and Bcl-2 genes. The antiapoptosis proteins, Bcl2 and $BclX_{L/S}$ were downregulated by IFN-γ, and the apoptotic protein Bak was upregulated in colorectal adenocarcinoma cell (64).

3.6 Role of cytkokine in inhibition of erythropoiesis

The majority of *in vitro* data on the potential role of cytokines in the pathogenesis of ACD in chronic inflammatory disorders have been obtained in rheumatoid arthritis (RA). Many cytokines involved in chronic acute phase response an inhibitory activity on erythroid colony formation *in vitro*. The cytokine that have a major inhibitory effect on red cell precursors in the bone marrow. Transforming growth factor beta (TGF-β) inhibits the earliest erythroid precursors and BFU-E both directly by inhibiting the activity of IL-3. The inhibitory effect of TNF-α on the growth of BFU-E and CFU-E is directly, at least partly mediated by the IFN-γ

produced by bone marrow stromal cells (65). Likewise indirect is the inhibitory effect of IL-1 on CFU-E generation and is mediated by the IFN-γ produced by T cells (66). The cytokines that negatively regulate erythroid precursors act synergistically, as consistently reported for IL-1 and TNF-α (67). Circulating TNF-α is elevated in RA (68) and IL-1β serum levels are significantly increased in RA with ACD as compared to RA without anemia (69). Moreover, it was also reported that in β-thalassemia/HbE showed increase level of cytokines include TNF-α, IL-1β and IFN-γ correlated with their clinical symptom, suggesting the possibility that cytokines might contribute to the inhibit erythropoietic activity (70-72).

3.7 Inducible nitric oxide synthase and nitric oxide production.

Nitric oxide (·NO) is an important intracellular and intercellular signaling molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in cardiovascular, nervous and immunological system. ·NO has contrasing role in living organisms. It acts as a biological mediator similar to neurotransmitters in the neuronal system, can regulate blood vessel tone in vascular systems, and is an important host defence effector in the immune system. On the other hand, it is a free oxygen radical and can act as a cytotoxic agent in pathological processes, particularly in inflammatory disorders (73-74). ·NO is a short-lived free radical and a very small compound that diffuses freely within cells from its site of formation to its site of action. The biosynthesis of ·NO in the organism is carried out from L-arginine and molecular oxygen utilizing NADPH as an electron donor and using heme, FMN, FAD and tetrahydrobiopterin (H4B) as cofactors through a reaction that consumes five electrons. The overall reaction consists of a two step oxidative conversion of L-arginine to ·NO and L-citrulline via Nω-hydroxy-L-arginine (NOHarginine) as an intermediate, with monooxygenase I and monooxygenase II, each step representing a mixed-function oxidation (Figure.8) (73, 75). The production of ·NO in the body is catalysed by a family of enzymes called nitric oxide synthases (NOSs) (130–160 kDa). NOS enzymes consist of different subtypes depending on the tissue type, although all share between 50–60% sequence homology (73). Currently, at least three distinct isoforms of NOS have been isolated: eNOS (endothelial NOS, NOS

I), iNOS (inducible NOS, NOS II) and nNOS (neuronal NOS, NOS III). nNOS and iNOS are soluble whereas eNOS is membrane bound, with its N-terminal myristoylated (76). Two enzymes, eNOS and nNOS isoforms, designated as constitutive NOS, are constantly present in resting cells, and are activated by calcium and calmodulin (CaM) (73). Upon demand by a signal molecule, ·NO is synthesized in low concentrations by constitutive NOS, binds to heme iron of soluble guanylate cyclase to yield the second messenger cGMP, which in turn modulates an array of mediators, including various ion channels, phoshodiesterases and protein kinases, decreasing intracellular calcium levels, and allows smooth muscle to relax (77). Stimulation of soluble guanylate cyclase by ·NO also inhibits platelet aggregation and vessel wall adhesion of platelets (78). The third isoform, inducible NOS (iNOS), is not present in resting cells but can be induced by immunostimulatory cytokines, bacterial products or infection in a number of cells, including endothelium, hepatocytes, monocytes, mast cells, macrophages and smooth muscle cells. It generates ·NO independently of intracellular calcium concentrations (73, 74). There is some evidence of a different NOS enzyme, mitochondrial NOS (mtNOS). Recently, the existence of mtNOS, its Ca2+ dependence, and its relevance for mitochondrial bioenergetics was reported in rat and mouse tissues, such as liver, thymus, skeletal muscle, heart, and brain (79, 80). Upon uptake of Ca2+ mtNOS is stimulated, peroxynitrite (ONOO-) is formed, and Ca2+ is subsequently released from intact mitochondria, suggesting a feedback loop which prevents overloading of mitochondria with Ca2+ (81). Enhanced ·NO production and ONOO- formation by mtNOS also lead to protein nitration in the mitochondrial matrix during mitochondrial dysfunction and contractile failure (82). However, the regulation of NOS isoform is still unclear (83).

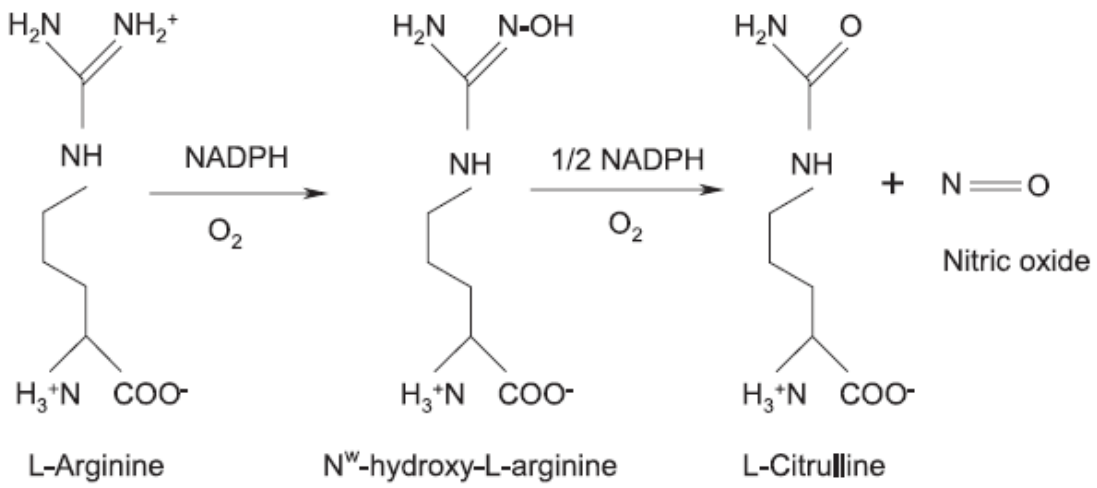


Figure 8. The reaction of ·NO synthesis from L-arginine. ·NO is synthesized endogenously by the conversion of L-Arginine to L-citruline (2).

·NO is not only synthesized enzymatically, but can also be produced nonenzymatically from nitrite at low pH under reducing conditions. Nonenzymatic ·NO production may play a role in similar biological events as when ·NO is generated from L-arginine by NOS enzymes, such as in the stomach, on the surface of skin, in the ischemic heart, and in infected nitrite-containing urine, but its importance in regulation of iNOS activity and ·NO production is unclear (84, 85). The overproduction of ·NO independently of intracellular calcium concentrations is due to iNOS that can result in either protective or damaging effects, although activation of ·NO production depends on the cell type. Many cell types can express iNOS for their function in host defense against microbial and viral pathogens (86), leading to the formation of ·NO radicals or S-nitrosothiols or ONOO- in the host cell or in the microbe itself. In addition to the protective effects of iNOS, iNOS expression in macrophages is activated by particular inducers, participating in the pathology of inflammatory diseases including atherosclerosis, rheumatoid arthritis, diabetes, septic shock, transplant rejection, and multiple sclerosis, leading to cell death (87-89).

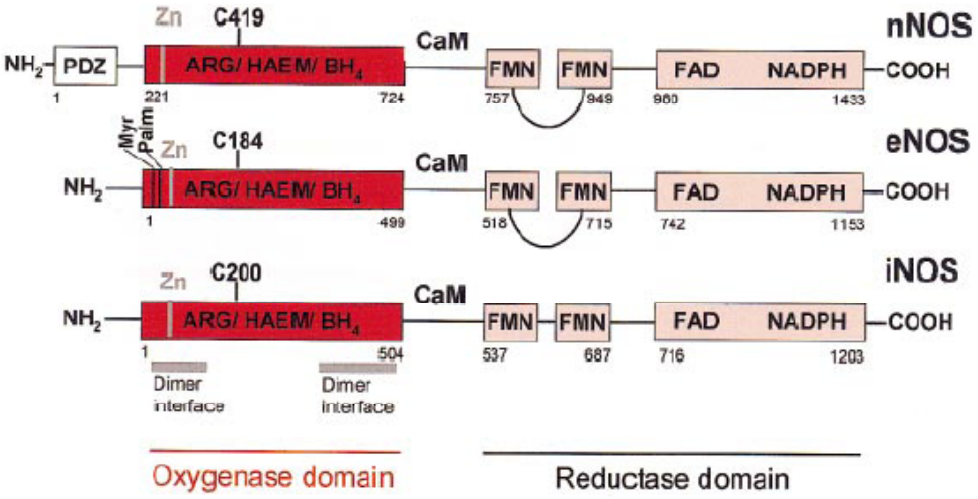


Figure 9. Structure of nitric oxide synthase (2).

Higher concentrations of NO produced by iNOS interact with thiol groups or transition-metal containing proteins and can alter protein function or initiate gene expression to protect cells. There is a continuous shift at even higher concentrations of NO towards cell damage or apoptosis, with other factors in the microenvironment of a cell critically influencing the final outcome (91). Two important observations were reported by different groups when working on iNOS expression. First, lipopolysaccharide (LPS) and interferon-γ (IFN-γ) are strong inducers of iNOS in monocytes, whereas the cytokines TNF-α and IL-1β have, if any, only marginal effects. In contrast, most tissue-derived cells including hepatocytes, mesangial cells, vascular smooth muscle cells, myocytes and pancreatic b-cells, to name just a few, are more sensitive to the induction of iNOS when challenged with TNF-α or, in particular, with IL-1β. In nearly all cell types, LPS, IFN-γ, IL-1β and TNF-α enhance iNOS expression synergistically. Second, rodent cells can more easily be triggered to express iNOS than human cells. For example, IL-1β is a strong stimulus for iNOS transcription in rat mesangial cells (92), whereas in human mesangial cells considerable iNOS expression occurs only when combinations of cytokines were administered as a cocktail (93). Conflicting results were obtained concerning the role of cyclic AMP (cAMP) in iNOS expression. Whereas in rat vascular smooth muscle cells and rat mesangial cells, cAMP is a strong inducer of iNOS mRNA (94, 95), cAMP reduces iNOS expression in other rodent cells. Recently, other stimulators of iNOS expression such as IL-2, IL-12, IL-18 and IFN-γ have been described (96).

Three main signalling pathways are thought to be responsible for iNOS expression. Induction of iNOS in murine macrophages is mediated by γ-activated sites and IFN-γ responsive elements in the iNOS promoter (97, 98). In rat mesangial cells, binding of the nuclear factor kB (NF-kB) at the appropriate site on the iNOS promoter is essential for the induction of iNOS by IL-1β (99). In contrast, endothelin-1 inhibits cytokine-induced iNOS expression without affecting NF-kB binding, suggesting additional mechanisms that are essential for cytokine-induced iNOS expression (100). For the induction of iNOS by cAMP, enhanced binding of the transcription factors CAAT/enhancer-binding protein (C/EBP) and cAMP-responsive element-binding protein (CREB) on the iNOS promoter has been reported (99). Many inflammatory diseases are accompanied by an increase in ·NO production and, in appropriate animal models, a beneficial action of iNOS inhibitors has been demonstrated. Therefore, many groups are currently studying physiological or synthetic agents that inhibit cytokine-induced iNOS expression at the transcriptional level (101). As a common theme, cytokine-induced iNOS expression is inhibited by agents that diminish activation or binding of NF-kB. This has been shown for a variety of pharmacological substances, including dithiocarbamates (92), dexamethasone and cyclosporin A (102). These novel therapeutic approaches may provide new methods for treating inflammatory diseases.

However, it has not been investigated in cytokine induced iNOS expression and ·NO production in erythroid progenitor cells. In this study, we would like to study the effect to cytokine include IL-1β, TNF-α and IFN-γ on iNOS expression and ·NO production. Its may play role in erythroid progenitor cells apoptosis in β-thalassemia/HbE and anemia of chronic disease.

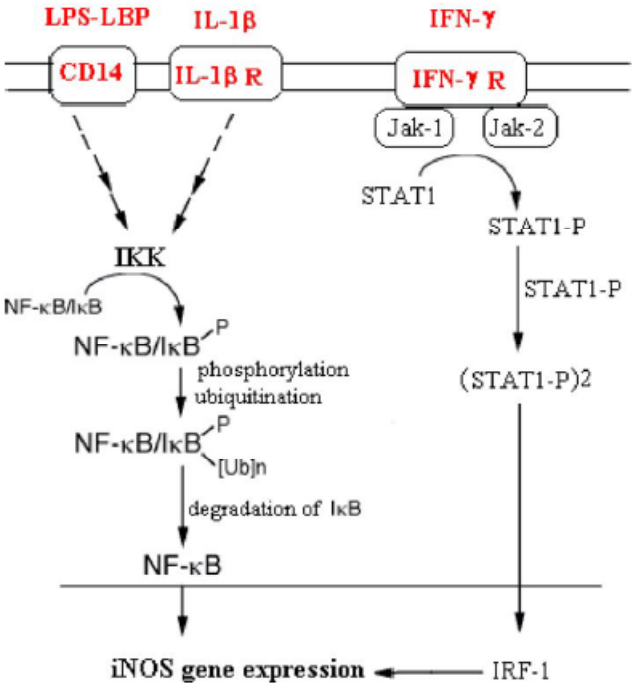


Figure 10. Activation of NF-kB and STAT signaling pathway by LPS, IL-1β and IFN-γ (2).

3.8 Nitric oxide and apoptosis

·NO is a pleiotropic mediator and signaling molecule involved in regulating cell number. ·NO shows opposite effects depending on its concentration and cell type (103).

3.8.1 ·NO as a pro-apoptotic inducer

Since ·NO is enzymatically synthesized from L-arginine in macrophages, the immunological function of ·NO revealed the induction of cytotoxicity against tumor cells and surrounding tissues. High concentrations of ·NO induce cell death in several cell types. These include macrophages, thymocytes, pancreatic islets, certain neurons, and tumor cells. Although the precise mechanism that determines the cellular sensitivity against ·NO-induced apoptosis are not clearly elucidated, the proapoptotic effects of ·NO on these cells seem to be independent (but not all) of the cGMP accumulation through the activation of soluble guanylate cyclase. The factors affecting

cell-specefic sensitity to ·NO mediated apoptosis can be associated with the redox state within the cells, activation of the apoptotic signaling cascade (such as cascases), the mitochondrial cytochrome c release, or regulation of cell survival and apoptotic gene expression. The induction of apoptosis often requires exposure to high levels of exogenous ·NO donors, which may overwhelm the natural protective mechanism of cells. This leads to the activation of the apoptotic-signaling pathway. Such toxic levels of ·NO may have limited relevance to the *in vivo* situation. Furthermore, the threshold of the ·NO level triggering apoptosis is different from one cell to the other (104).

3.8.2 ·NO as an anti-apoptotic modulator

Although ·NO promotes apoptosis in some cells, it has been demonstates that ·NO displays anti-apoptotic properties in other cell types. These include hepatocytes, human B lymphocytes, endothelial cells, splenocytes, eosinophils, and P12 cells. In the animal model, the lipopolysaccharide (LPS) induced hepatic apoptosis increased by the administration of NOS inhibitors. The administration of liver-specific ·NO donor almost completely suppressed the caspase-3-like activity, as well as the massive hepatic apoptosis that is induced by the administration of TNF-α plus D-galactosamine. In addition, ·NO can protect some cells from apoptosis that is induced by many different types of stimuli, such as TNF-α, oxidative stress, serum-deprivation and anoxia. This evidence shows that ·NO inhibits apoptosis both *in vitro* and *in vivo* in certain cell types and experiment conditions (105). The biochemical mechanism underlying the ·NO-mediated anti-apoptotic effects may be cell-type specific with multiple pathways. For example, ·NO blocks apoptosis in PC12 cells, predominantly via the ·NO/cGMP/PKG pathway, and inhibits hepatocyte apoptosis, both through cGMP dependent interruption of apoptotic signaling and direct inhibition of caspase activity (105). ·NO that is generated either by the .NO donor and NOS can block apoptosis. For example, endothelial cells, low level of ·NO from eNOS blocks TNF-α induced and serum-deprived apoptosis. Also, a high level of ·NO from iNOS transfection and ·NO donor inhibits LPS induced and serum-deprived apoptosis. The precise mechanisms for the ·NO-mediated inhibition of apoptosis have not been clearly elucidated. However, a series of molecular targets such as iron-sulfur complexes, soluble guanylate cyclase, caspases, glutathione in varying in the cell types and

apoptotic stimuli for ·NO were identified. They can suppress apoptotic cell death either by indirect or direct interaction with the apoptotic-signaling cascade (105).

CHAPTER IV
MATERIALS AND METHODS

4.1Materials

4.1.1 Reagents and instruments

4.1.1.1 Reagents for erythroid cell culture

- Iscove's modified Dulbecco's medium; IMDM (GIBCO^{TM}, Invitrogen Corporation)
- Fetal Bovine Serum; FBS (GIBCO^{TM}, Invitrogen Corporation)
- Penicillin-Streptomysin (GIBCO^{TM}, Invitrogen Corporation)

4.1.1.2 Reagents for analysis

- Trypan blue (Sigma-Aldrich, USA)
- Annexin V-FITC Apoptosis detection (BD Bioscience, USA)
- Griess Reagent System (Promega, USA)
- Wright-Giemsa (Merck, Germany)
- Trizol reagent (Invitrogen corporation)
- Superscript^{TM}III First-Strand Synthesis System for RT-PCR (Invitrogen corporation)
- iQ^{TM}SYBR®Green Supermix (Bio-Rad, USA)

4.1.1.3 Instruments

- Hemocytometer
- Hand counter
- Centrifuge (Hettich Zentrifuger universal 320R, Germany)
- Light microscope (Olympus, Japan)
- FACSCalibur E6361 Flow cytometer (Beckton-Dickinson, NJ)
- CO_2 incubator (SL shel lab, USA)

- Automatic pipette
- Microflow advance Biosafety Cabinet-class II (Science Tech)
- Water bath (JulaboTW8, Germany)
- Microplate Reader Multimode detector DTX800 (Beckman Coulter, USA)
- DNAEngine®Peltier Thermal Cycler (Bio-Rad, USA)
- UV-2450 UV-visible Spectrophotometry (Shimadzu Corporation, Japan)
- iCycleriQ® Multicolor Real-time PCR Detection system (Bio-Rad, USA)

4.1.1.4 Glassware, Plastic ware and Supplies

- 6 wells cell culture plates (Corning Incorporation, USA)
- 24 wells cell culture plates (Corning Incorporation, USA)
- 96 wells cell culture plates (Corning Incorporation, USA)
- 25 cm^2 culture flasks (Corning Incorporation, USA)
- 15 ml centrifuge tube (Corning Incorporation, USA)
- 50 ml centrifuge tube (Corning Incorporation, USA)
- 5 ml polystyrene round button tube (Flacon, France)
- Beaker, glass, 50, 100, 250, 500 and 1000 ml
- Bottle, glass, 250, 500 and 1000 ml
- Cylinder 250, 500 and 1000 ml
- Glass slide and cover glass
- Microcentrifuge tube 1.5 ml (Axygen Incorporation, USA)
- PCR tube 0.2 ml thin well, Flat cap (Axygen Incorporation, USA)
- Millipore filter 0.22 µm
- Pipette tip 20, 200 and 1000 ml
- Seropipette 5, 10 ml
- 70% alcohol

4.1.2 Cytokines

4.1.2.1 Interleukin-1beta ; IL-1β (Chemical international)

Interleukin-1beta (IL-1β) is a potent immune-modulator that mediates a wide range of immune and inflammatory responses including the activation of B and T cells. Human IL-1β is a 17.3 kDa protein containing 153 amino acid residues

4.1.2.2 Tumor necrosis factor-alpha ; TNF-α (Chemical international)

TNF-α is a potent lymphoid factor with exerts cytotoxic effects on a wide range of tumor cells and certain other target cells. The mature form of human TNF-α has 157 amino acid residues.

4.1.2.3 Interferon-gamma ; IFN-γ (Chemical international)

IFN-γ is a lymphoid factor which possesses potent anti-viral activity. It has also been shown to stimulate macrophage and NK cells. Human IFN-γ is a 16.7 kDa protein containing 143 amino acid residues.

4.1.3 iNOS inhibitor S-Methyisothiourea Sulfate ; SMT (Chemical international)

SMT is a highly selective, cell peameable inhibitor of inducible nitric oxide synthase (iNOS).

4.2 Methods

4.2.1 CD 34 positive cell selection and Erythroid cell culture

CD 34 positive cell were selected by EasySep® kit and then cultured in Iscove's modified Dulbecco's medium (IMDM) supplement with 2 U/ml erythropoietin, 15 % fetal bovine serum (FBS), 10 ng/ml stem cell factor, 15% human AB serum and 10 ng/ml interleukin-3 along with 2% penicillin-streptomycin. Cells were grown at 37 °C in humidified atmosphere of 5% CO_2 for 14 days.

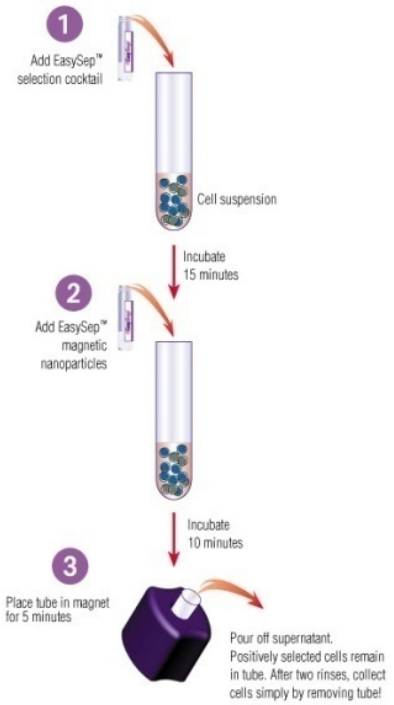


Figure11. General EasySep procedure

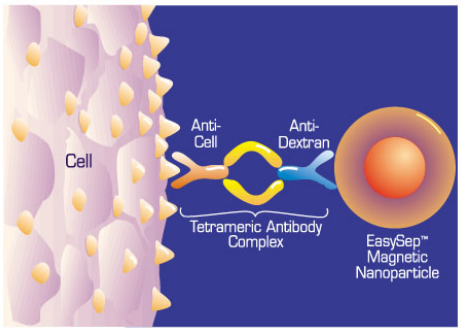


Figure12. Schematic drawing of EasySep® magnetic labeling of human cells

4.2.2 Cytokines and inhibitor treatment

4.2.2.1 Cytokine treatment

Erythroid progenitor cells were treated with 2, 20, 40 ng/ml of IL-1β, TNF-α, and IFN-γ, then cultured for 14 days at 37°C in a humidified atmosphere of 5% CO_2. Untreated cell were used as control cell.

4.2.2.2 iNOS inhibitor treatment

Erythroid progenitor cells were treated with 2, 20, 40 ng/ml of

IL-1β, TNF-α, and IFN-γ and then immediately treated with 1 ng/ml of SMT, then cultured for 14 days at 37°C in a humidified atmosphere of 5% CO_2.

4.2.3 Iron study

3 ml of whole blood was collected in clotted blood tube. Serum was separated and stored at -70˚C for measuring serum iron, serum ferritin, total iron binding capacity and transferrin saturation.

4.2.4 Total cell count and cell viability assay

Trypan blue is large anionic dye that belongs to the group of azo dyes. Trypan blue is traditionally used as component of polychromatic stains, e.g. for demonstration of collagenous connective tissue. Trypan blue is a vital stain recommended for use in estimating the proportion of viable cells in a population. The reactivity of this dye is based on the fact that the chromophore is negatively charged and does not react with the cell unless the membrane is damaged. Staining facilities the visualization of cell morphology. Live (viable) cells do not take up the dye and dead (non-viable) cells do.

4.2.4.120 µl of cell susupension was transfered into appendrof.

4.2.4.2 Cells were mixed with 20 µl of trypan blue solution in ratio 1:1 then allow the cell suspension mixture standed at least 5 minutes.

4.2.4.3 The mixture was transfered to chamber of the hemocytometer. Carefully touched the edge of cover chamber with the pipette tip and allowed each chamber to fill by capillary action.

4.2.4.4 Cells were counted on top and left touching middle line of the perimeter of each square. Do not count cells touching the middle line at the bottom and right sides. Viable cells exclude trypan blue, while non-viable cells will stain blue due to trypan blue uptake.

4.2.4.5 Cells were counted in each squares of the hemocytometer represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. This is the conversion factor for the hemocytometer.

4.2.4.6 Since 1 cm^3 is approximately 1 ml, the subsequent cell

concentration/ml and total cell number was determined using the following calculation.

$$\text{Total cells} = \frac{\text{cell count x dilution factor x volume}}{\text{Area}}$$

$$\text{Cell viability (\%)} = \frac{\text{number of unstained (viable) cells}}{\text{Total cells}} \times 100$$

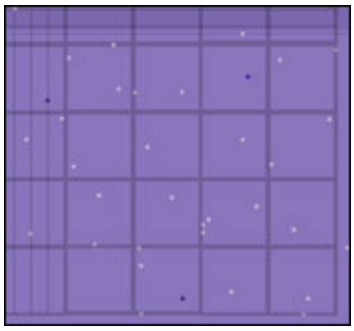


Figure 13. Hemocytometer (counting chamber)

4.2.5 Cell apoptosis assay by flow cytometry

Erythroid progenitor cells apoptosis were measured using annexinV-FITC glycophorin A-PE staining followed by FACS analysis. In early apoptosis phase, plasma membrane phospholipid phosphatidylserine (PS) which will be translocated from the inner to the outer leaflet and then will be detected using the green fluorescent protein Annexin-V. Glycophorin A-PE staining was also used simultaneously for erythroid marker.

4.2.5.1The cells ($1x10^5$ cells/ml) were harvested by centrifugation at 12,000 rpm for 5 minutes and then cell pellets were mixed with PBS and centrifugation at 12,000 rpm for 5 minutes.

4.2.5.2 Cell pellets were added with 1X binding buffer and stained with 2 µl Annexin V-FITC and 10 µl of glycophorin A, then left at RT for 15 minutes in dark.

4.2.5.3The stained cells were analyzed by flow cytometry.

4.2.5.4 The number of cell stained with Annexin V-FITC and glycophorin A-PE positive was measured for percentage of cell apoptosis.

4.2.6 Nitric oxide production assay

Griess assay is base on detection of total concentrations of NO stable and products, nitrite (NO_2^-), chemical reaction shown in Figure 14. This assay relies on a diazotization reaction of chemical reaction, which uses sulfanilamide and N-1-napthylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Concentrations of nitrite will be measured using a spectrophotometer.

A nitrite standard reference curve must be prepared for each assay for accurate quantitation of NO_2^- levels in experimental samples. Prepare reference curves in the same medium for experimental samples (Figure 15).

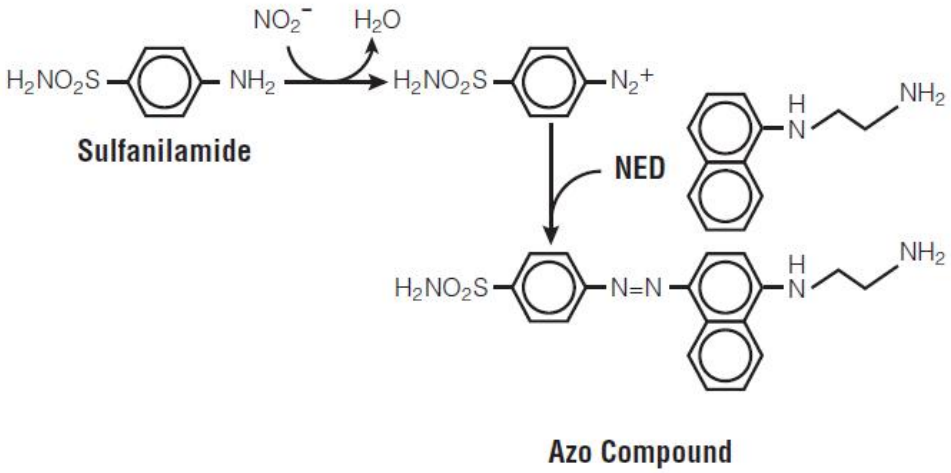


Figure 14. Chemical reactions involved in the measurement of NO_2^- using the Griess Reagent System

4.2.6.1 Preparation of nitrite standard (reference) curve.

- 1 ml of 100 µM nitrite solution was prepared by diluting the provided 0.1 M nitrite standard 1:1,000 in the medium.

- 3columns in the 96 well plate was designated for nitrite standard reference curve and dispensed 50 µl of the medium into wells in rows B-H

- 100 µl of the 100 µM Nitrite standard solution was added to the remaining 3 wells in row A and immediately performed 6 serial two-fold dilutions (50µl/well) in triplicate down the plate to generated the nitrite standard (reference) curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56µM), then discarded 50 µl from 1.56 µM set of wells. Do not add any nitrite standard solution to the last set of wells (0µM)

4.2.6.2 Nitrite Measurement

- The Griess assay was initiated by harvest the cells and centrifugation at 12,000 rpm for 5 minutes at room temperature

- 50 µl of each supernatant was added to wells in triplicate.

- Multichannel pipettor was used to dispense 50 µl of the sulfanilamide solution to all experimental samples and wells containing the dilution series for the nitrite standard (reference) curve, incubated 10 minutes at room temperature and kept plate in dark.

- Multichannel pipettor was used to dispense 50 µl of the NED solution to all wells and incubated 10 minutes at room temperature. Then a purple color was begun to form immediately.

- The absorbance at 570 nm was recorded within 30 minutes in a plate reader.

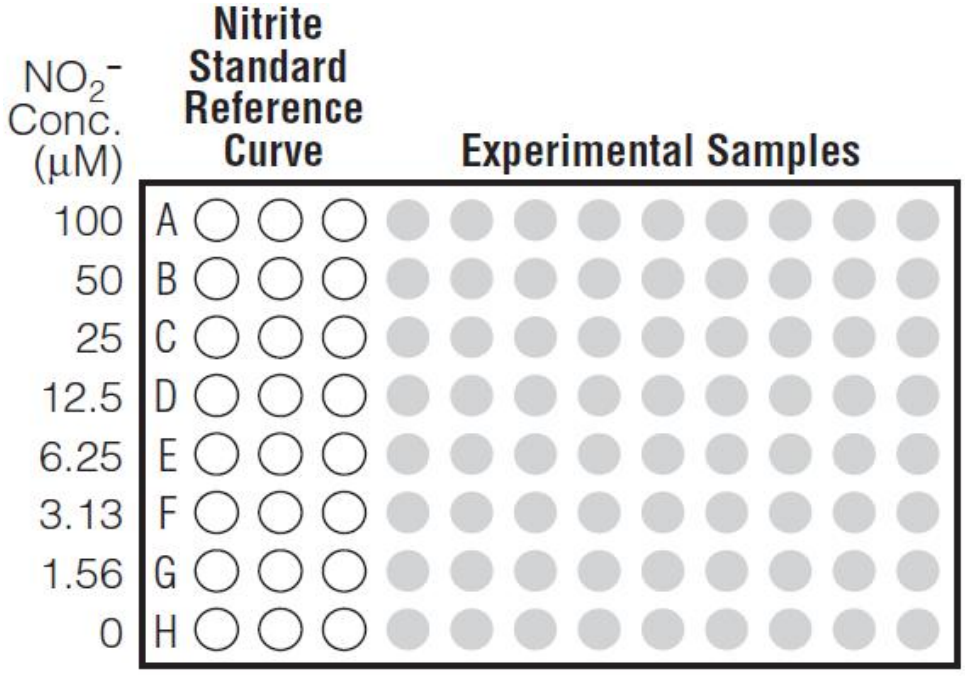


Figure 15. Suggested plate format for the nitrite standard reference curve.

4.2.7 RNA extraction

Trizol reagent combines phenol and guanidine thiocyanate solution to facilitate the immediate and most effective inhibition of RNase activity. A biological

sample is homogenized or lysed in Trizol Reagent and the homogenate is separated into aqueous and organic phase, DNA in the interphase and proteins in the organic phase. RNA is precipitated from the aqueous phase by addition of isopropanol, washed with ethanol and solubilized.

4.2.7.1 The cell suspension was harvested by centrifugation at 3,500 rpm for 5 minutes at room temperature.

4.2.7.2 The medium was removed and cell pellets were resuspend in 1 ml of sterile ice cold PBS and centrifuge at 3,000 rpm for 5 minutes.

4.2.7.3 The pellet was resuspended in 1000 µl of Trizol reagent and mixed well by repetitive pipetting.

4.2.7.4 200 µl of chloroform were added and sharked tubes vigorously by hand for 15 seconds. Then incubated at RT for 2-3 minutes.

4.2.7.5 The sample were centrifuged at 12,000 rpm for 15 minutes at 4°C and transferred the upper phase (colorless) to new microfuge tube.

4.2.7.6 500 µl of ice-cold isopropanol were added and mixed, then the sample was stored at -20°C for 10 minutes.

4.2.7.7 The sample was centrifuged at 12,000 rpm for 10 minutes at 4°C and discarded supernatant.

4.2.7.8 Pellet was washed with 1 ml of cold 70% ethanol and centrifuged at 7,500 rpm for 5 minutes at 4°C and then discard supernatant.

4.2.7.9 Ethanol was carefully removed from tube and the RNA pellet was briefly dried for 5 minutes.

4.2.7.10 RNA was resuspended in 50 µl of RNAse free distilled water and incubated at 50°C for 10 minutes to dissolve RNA.

4.2.8 RNA quantification

The concentration of RNA sample can be measured by the use of UV spectrophotometer. RNA absorbs UV light very efficiently making it possible to detect and quantify. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm. The absorbance of 1 unit at 260 nm in corresponds to 40 µg of RNA per ml.

4.2.8.1 Lens paper was used to clean the surfaces of the cuvette and rinsed the cuvette with DW.

4.2.8.2 0.6 ml of D.W. was added to the cuvettes as a blank.

4.2.8.3 Absorbance was recorded at 260 and 280 nm.

4.2.8.4 The sample was prepared in 1:50 dilution.

4.2.8.5 After the machine was read the blank, the cuvette was removed.

4.2.8.6 0.6 ml of sample was added to the cuvettes, the absorbance was recorded at 260 and 280 nm.

4.2.8.7 The concentration of RNA sample was calculated.

RNA concentration (μg/ml) = (OD_{260}) x (40 μg RNA/ml)/ (1 OD_{260} unit)

4.2.9 First-Strand cDNA Synthesis

The SuperScript^{TM} III First-Strand Synthesis System for RT-PCR is optimized to synthesized first-strand cDNA from purified poly (A)+ or total RNA. RNA targets from 100 bp to >12 kb can be detected with this system. The amount of starting material can vary from 1 pg to 5 μg of total RNA. cDNA synthesis is performed in the first step using poly (A)+ selected RNA primed with oligo (dT). Oligo (dT), a more specific priming method, is used to hybridize to 3' poly (A) tails, which are found in the majority of eukaryotic mRNAs. In the second step, PCR is performed in a separate tube using primers specific for the gene of interest.

4.2.9.1 The cDNA synthesis master mix was performed following this protocol.

Component	Amount
10X RT buffer	2 μl
25 mM $MgCl_2$	4 μl
0.1 M DTT	2 μl
RNaseOUT (40 U/μl)	1 μl
SuperScript III RT (200 U/μl)	1 μl
Total	10 μl

4.2.9.2 10 µl of cDNA Synthesis mix was added to each RNA mixture, mixed gently, and then incubated at 50°C 50 minutes, after that incubated at 85°C for 5 minutes.

4.2.9.3 1 µl of RNase H was added to each tube and incubated at 37°C for 20 minutes.

4.2.9.4 cDNA synthesis reaction was stored at -70°C or used for PCR immediately.

4.2.10 Real-time PCR

The introduction of the new procedure based on fluorescence kinetic RT-PCR enables quantification of the PCR product in "real-time". This sensitive and accurate technique measures PCR product accumulation during the exponential phase of the reaction. The technique is much faster than the previous endpoint RT-PCR as it is designed to provide information as rapidly as the amplification process itself, thus requiring no post PCR manipulation.

4.2.10.1 SYBR Green

SYBR Green is a DNA-binding dye that incorporates into dsDNA. It has an undetectable fluorescence when it is in its free form, but once bound to the dsDNA it starts to emit fluorescence. Its greatest advantage is that it can be used with any pair of primers for any target, making its use less expensive than that of the probe. However, specificity is diminished due to the risk of amplifying nonspecific PCR products. Indeed, SYBR Green I binds to any dsDNA, detecting not only the specific target, but also nonspecific PCR products and primer dimers.

- PCR mixture was prepared following this protocol.

Component	Amount
iQ SYBR Green Supermix	25 µl
Sense primer (10 µM)	2 µl
Antisense primer (10 µM)	2 µl
cDNA from control RNA	2 µl
DEPC-treated water	19 µl
Final volume	50 µl

- The reaction components were centrifuged briefly.

- A real-time PCR was performed on iCycleriQ® Multicolor Real-time PCR Detection system for 35 cycles following this protocol.

Cycle 1: (1X)
Step 1: 95.0 °C for 3 min
Cycle 2: (40X)
Step 1: 95.0 °C for 30 sec
Step 2: 62.5 °C for 30 sec
Step 3: 72.0 °C for 1.5 min
Cycle 3: (1X)
Step 1: 95.0 °C for 1 min
Cycle 4: (1X)
Step 1: 55.0 °C for 1 min
Cycle 5: (81X)
Step 1: 55.0 °C for 10 sec

Primers	Sequences (5'→3')	Product length (bp)
iNOS-F	GCTGTATTTCCTTACGAGGCGAAGAA	257-bp
iNOS-R	CTTGTTAGGAGGTCAAGTAAAGGGC	
β_2-microglobulin-F	CATCCAGCGTACTCCAAAGA	164-bp
β_2-microglobulin-R	GACAAGTCTGAATGCTCCAC	

4.2.11 Statistical Analysis

The experiments were performed compare the effect among control (untreated cell) and treated cells and the results were expressed as mean ±S.D. the statistical analysis was performed by t-test significant statistic difference at *p-value*<0.05.

CHAPTER V
RESULTS

5.1 Hematological data

In this study, samples were classified into three groups included healthy subjects, β-thalassemia/hemoglobin E and anemia of chronic disease. The hematological data were analyzed and calculated the mean for each parameter of blood incidies WBC, RBC, Hb, Hct, MCV, MCH, MCHC, Plt and RDW. The data was shown in Table 3-5.

Blood indicies of healthy subjects were in reference range. β-thalassemia/ Hb E showed hemoglobin level, mean corpuscular volume and mean corpuscular hemoglobin less than reference range. In anemia of chronic disease, hemoglobin level less than reference range but the mean corpuscular volume and mean corpuscular hemoglobin were in reference range according to the criteria of both diseases.

Table 3. Blood indicies of healthy subject

Sample No.	Age/ Sex	WBC ($x10^3$/ul)	RBC ($x10^6$/ul)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (pg)	Plt ($x\ 10^3$/ul)	RDW (%)
1	23/F	6.7	4.6	12.9	39.5	86.6	28.3	32.7	220	12.9
2	25/M	5.2	5.1	15.7	47.6	92.7	30.5	32.3	172	14.3
3	28/F	5.7	4.3	12.7	39.4	90.9	29.4	32.3	313	12.2
4	25/F	6.7	4.3	13.6	39.9	92.4	31.3	33.9	288	11.3
5	36/M	6.7	5.2	15.8	47.4	90.7	30.3	33.4	214	12.1
average		**6.2**	**4.7**	**14.2**	**42.8**	**90.66**	**29.96**	**32.9**	**241**	**12.6**

Table 4. Blood indicies of β-thalassemia/hemoglobin E.

Sample No.	Age/ Sex	WBC ($x10^3$/ul)	RBC ($x10^6$/ul)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (pg)	Plt ($x\ 10^3$/ul)	RDW (%)
1	32/F	8.5	3.6	5.9	19.2	52.9	16.2	30.6	325	26.3
2	36/F	7.9	3.8	6.7	23.7	61.4	17.4	28.3	208	24.9
3	20/F	9.3	4.0	6.2	24.6	58.7	18.0	30.6	264	24.1
4	19/M	8.3	4.9	9.7	29.4	60.3	19.8	32.9	546	22.7
5	26/M	8.8	5.1	8.9	23.6	62.5	18.7	29.7	286	25.6
average		**8.6**	**4.3**	**7.5**	**24.1**	**59.2**	**18.0**	**30.4**	**325.8**	**24.7**

Table 5. Blood indicies of anemia of chronic disease.

Sample No.	Age/Sex	WBC ($x10^3$/ul)	RBC ($x10^6$/ul)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (pg)	Plt ($x\ 10^3$/ul)	RDW (%)
1	52/F	5.8	4.5	10.1	36.0	86.3	25.7	31.8	260	15.7
2	60/F	7.2	3.9	9.8	32.5	88.4	29.8	32.8	212	14.6
3	49/F	7.5	3.7	11.2	34.7	90.7	28.4	34.0	310	15.4
4	70/F	5.3	3.2	9.3	28.8	89.4	28.9	32.3	247	N/A
5	73/F	4.7	3.3	9.8	31.4	95.7	29.7	31.2	269	16.2
average		**6.1**	**3.7**	**10.4**	**32.7**	**90.1**	**28.5**	**32.4**	**259.6**	**15.5**

5.2 Iron study

A modification to the methods recommended by the International Committee for Standardization in Haematology was used to determine iron parameter by calorimetric assay. Iron status of three group of samples was shown in Table 6. There were a significant different the serum iron, total on binding capacity, transferrin iron saturation and serum ferritin between healthy subjects, β-thalassemia/hemoglobin E and anemia of chronic disease. In anemia of chronic disease the level of serum iron, TIBC and transferrin saturation less than reference range. The level of serum ferritin higher than reference range which related to the criteria of anemia of chronic disease.

Table 6. Indicies of the iron status (mean ±SD) in the healthy subjects, β-thalassemia/hemoglobin E and anemia of chronic disease.

Types of subjects	Serum iron (μmole/L)	Serum ferritin (ng/mL)	TIBC (μmole/L)	Transferrin saturation (%)
Healthy	28.2±6.08	111.5±94.05	45.7±4.53	61.45±7.14
β-thalassemia /HbE	11±2.21	416±547.58	32±2.13	34.53±7.52
ACD	28.4±4.47	1581±448.15	32.87±7.09	87.03±5.3

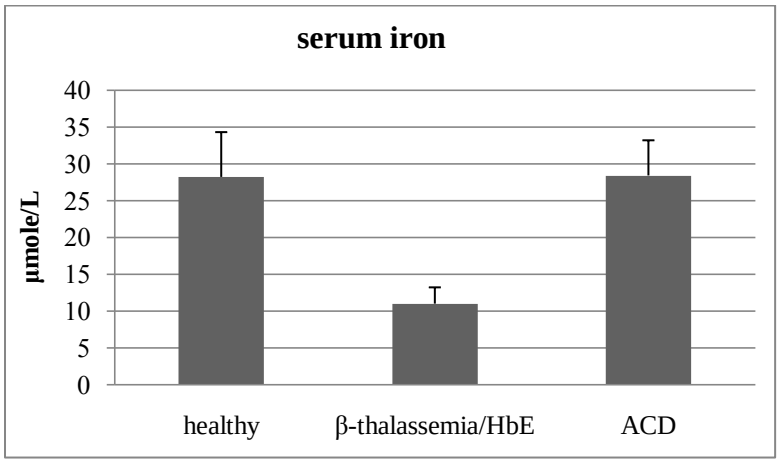


Figure 16. The level of serum iron in the healthy subjects, β-thalassemia/HbE and anemia of chronic disease.

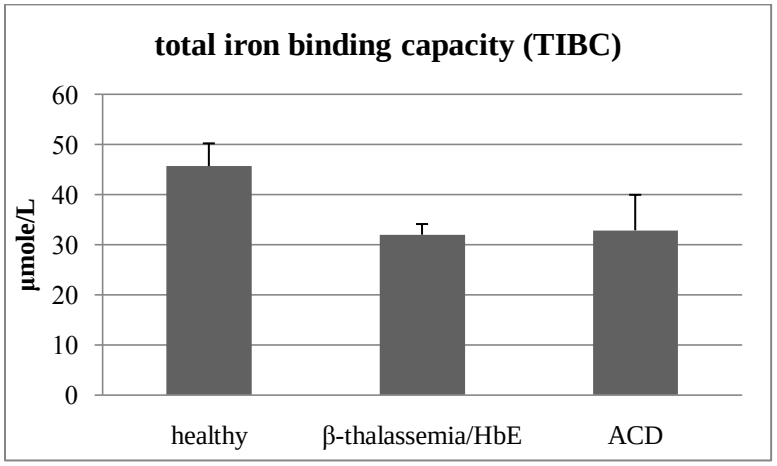


Figure 17. The level of total iron binding capacity in the healthy subjects, β-thalassemia/HbE and anemia of chronic disease.

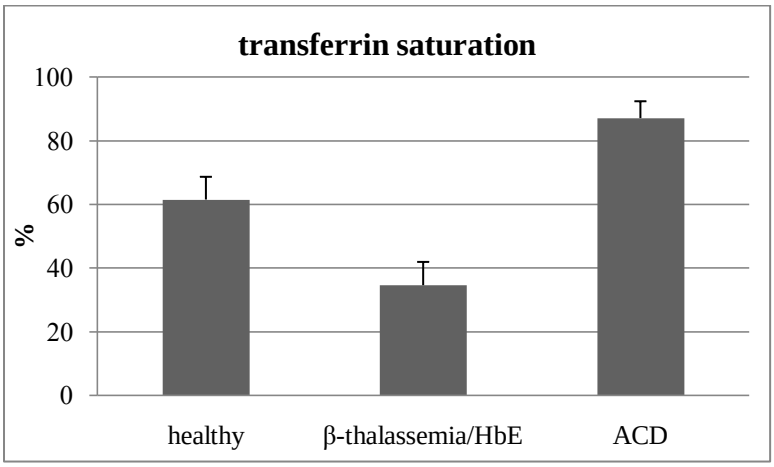


Figure 18. The level of trasferrin iron saturation of in the healthy subjects, β-thalassemia/HbE and anemia of chronic disease.

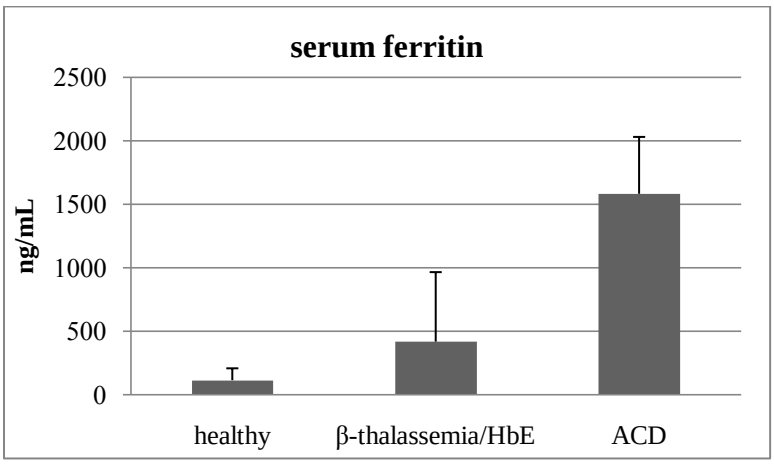


Figure 19. The level of serum ferritin in the healthy subjects, β-thalassemia/HbE and anemia of chronic disease.

5.3 Total cell count of erythroid progenitor cells.

Erythroid progenitor cells from healthy subjects, β-thalassemia/HbE and anemia of chronic disease (ACD) were used as a model of hematological diseases. Erythroid progenitor cells were cultured in Iscove Modified Dulbecco's Medium (IMDM) supplemented with 2U/ml erythropoietin, 10 ng/ml stem cell factor, 15% human AB serum, 10% fetal bovine serum, 10 ng/ml interleukin-3 and 2% penicillin-streptomycin. Cells were maintained in a humidified incubator at 37˚C and 5% CO_2. The total cells were counted at days 0, 3, 7, 10 and 14 on hemocytometer under light microscope.

At day 14, the total cell count of erythroid progenitor cells from healthy subjects, β-thalassemia/hemoglobin E and anemia of chronic disease were 241.5±25.9, 192.33±20.22 and 15.75±2.94, respectively. The total cells count of healthy subjects higher than β-thalassemia/hemoglobin E and anemia of chronic disease (ACD) (Figure 20).

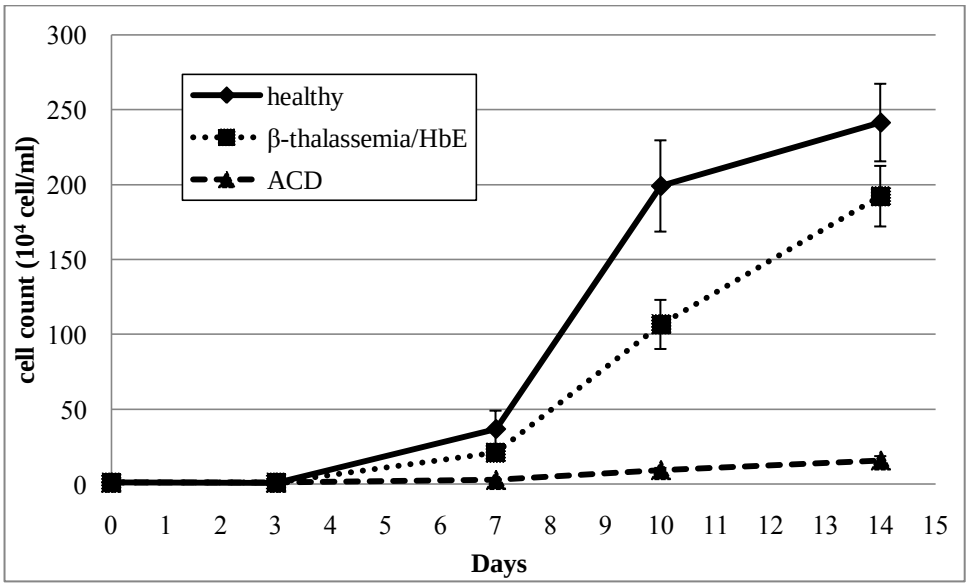


Figure 20. Mean of cell count from erythroid progenitor cells of healthy subjects, β-thalassamia/hemoglobin E and anemia of chronic disease.

5.4 Effect of cytokines on total cell count of erythroid progenitor cells

Erythroid progenitor cells from healthy subjects, β-thalassemia/HbE and anemia of chronic disease were cultured in erythroid condition and grown at 37°C in a humidified atmosphere of 5% CO_2 for 14 days. Interleukin-1beta (IL-1β), Tumor necrosis factor-alpha (TNF-α) and Interferon-gamma (IFN-γ) are proinflammatory cytokines that have been reported to induce apoptosis in several cells. We evaluated the effect of cytokines on total cell count of erythroid progenitor cells. Cells were cultured with or without various concentrations (2, 20 and 40 ng/ml) of interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ). The total cell count was measured by trypan blue staining on hemocytometer under light microscope at days 0, 3, 7, 10 and 14.

The results showed cell count was increased in time dependent manner. The highest cell count was found in day 14.

In healthy subjects, the total cell count of erythroid progenitor cells treated with 2, 20 and 40 ng/ml IL-1β at day 14 were $224x10^4$, $188x10^4$ and $200x10^4$ cell/ml, respectively. The total cell count of cells treated with 2, 20 and 40 ng/ml TNF-α were $198x10^4$, $173x10^4$ and $187x10^4$ cell/ml, respectively. The total cell count of cells treated with 2, 20 and 40 ng/ml IFN-γ were $173x10^4$, $162x10^4$, $152x10^4$ cell/ml, respectively. The lowest cell count was found in 40 ng/ml IFN-γ (Figure 21).

In β-thalassemia/hemoglobin E, the total cell count of erythroid progenitor cells treated with 2, 20 and 40 ng/ml IL-1β at day 14 were $132x10^4$, $71x10^4$ and $75x10^4$ cell/ml, respectively. The total cell count of cells treated with 2, 20 and 40 ng/ml TNF-α were $83x10^4$, $53x10^4$ and $59x10^4$ cell/ml, respectively. The total cell count of cells treated with 2, 20 and 40 ng/ml IFN-γ were $66x10^4$, $55x10^4$, $49x10^4$ cell/ml, respectively. The lowest cell count was found in 40 ng/ml IFN-γ (Figure 22).

In anemia of chronic disease, the total cell count of erythroid progenitor cells treated with 2, 20 and 40 ng/ml IL-1β at day 14 were $12x10^4$, $10x10^4$ and $11x10^4$ cell/ml, respectively. The total cells count of cell treated with 2, 20 and 40 ng/ml TNF-α were $10x10^4$, $6x10^4$ and $7x10^4$ cell/ml, respectively. The total cell count of cells treated with 2, 20 and 40 ng/ml IFN-γ were $5.2x10^4$, $4.8x10^4$, $3.9x10^4$ cell/ml, respectively. The lowest cell count was found in 40 ng/ml IFN-γ (Figure 23).

From the results indicated that cytokine treated cells had lower total cell count than untreated cell (control) in all group. Both of β-thalassemia/hemoglobin E and anemia of chronic disease had lower cell count than healthy subjects. The suitable concentration for each cytokine, which suppressed erythroid progenitor cell proliferation were 20 ng/ml IL-1β, 20 ng/ml TNF-α and 40 ng/ml IFN-γ. In addition, 40 ng/ml IFN-γ was not statistic significant different compared with 20 ng/ml IFN-γ (P>0.05).

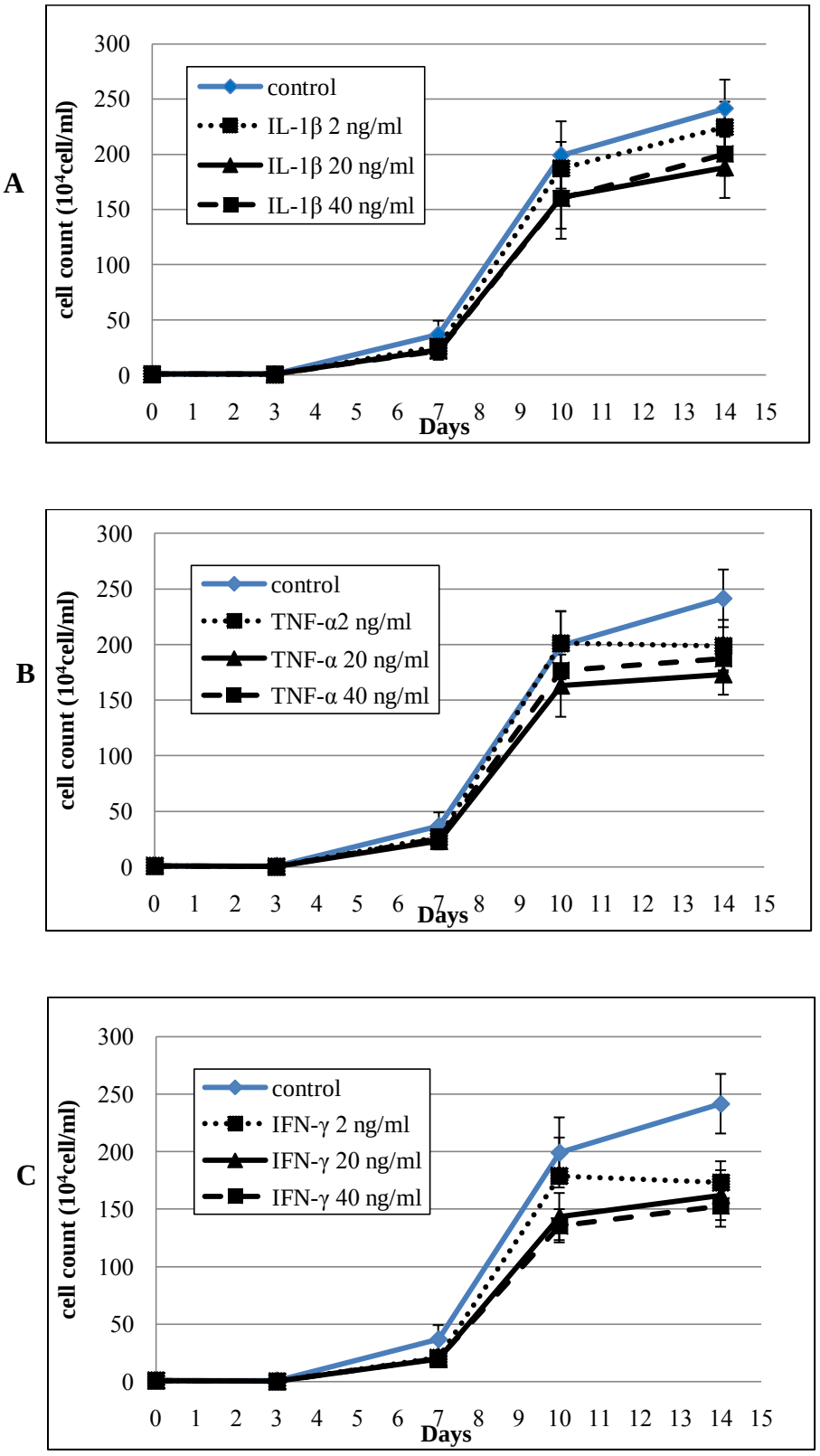


Figure 21. Mean cell count of erythroid progenitor cells from healthy subjects. Cells treated with various concentrations and time of IL-1β (A), TNF-α (B) and IFN-γ (C).

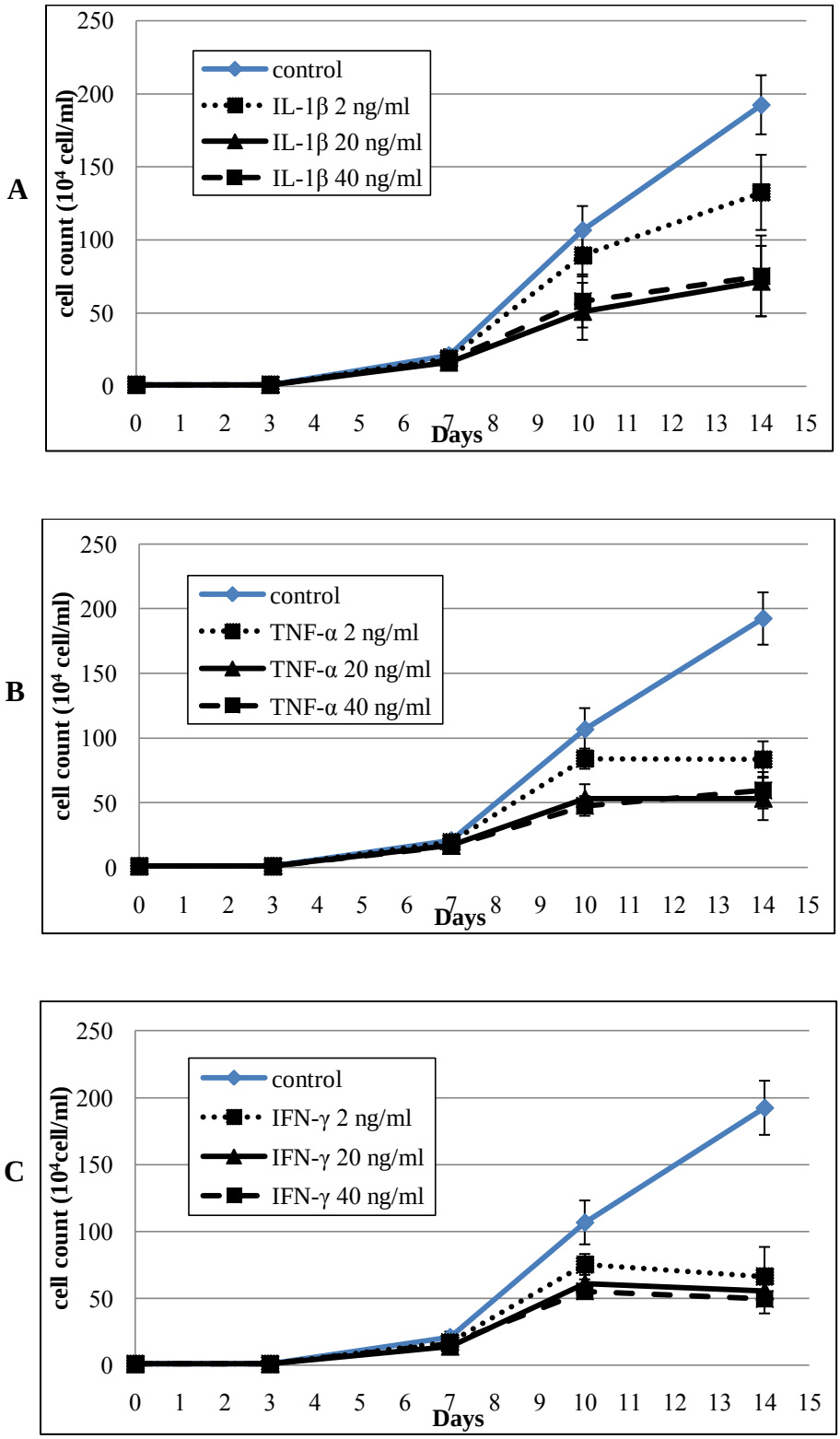


Figure 22. Mean cell count of erythroid progenitor cells from β-thalassemia/HbE. Cells treated with various concentrations and time of IL-1β (A), TNF-α (B) and IFN-γ (C).

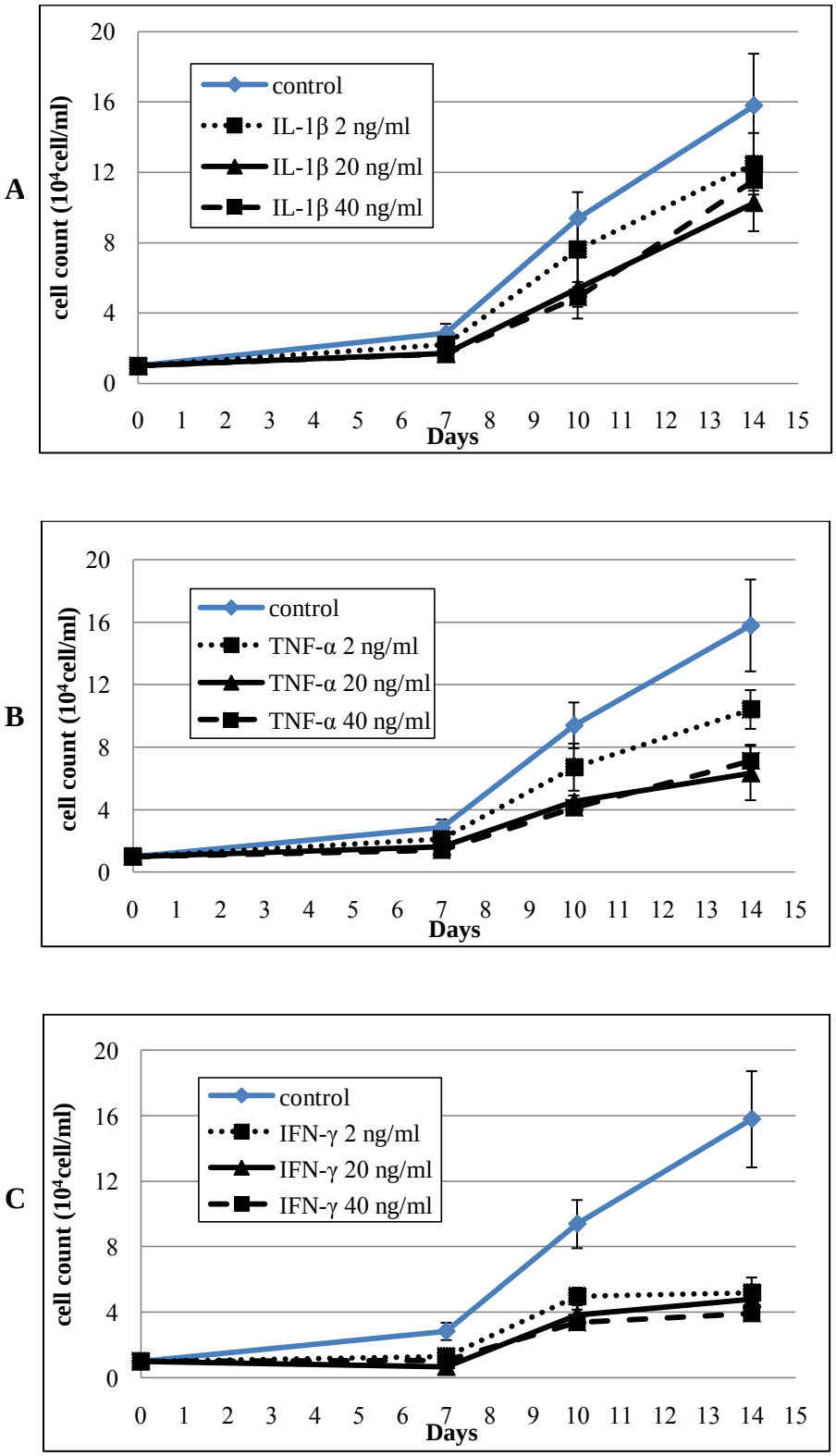


Figure 23. Mean cell count of erythroid progenitor cells from anemia of chronic disease. Cells treated with various concentrations and time of IL-1β (A), TNF-α (B) and IFN-γ (C).

5.5 Effect of cytokines on cell viability of erythroid progenitor cells

In order to investigate the effect of cytokines on reduction of cell viability in erythroid progenitor cells, cells were treated with various concentration of cytokines for 14 days. The percentage of cell viability was determined by trypan blue staining compared with untreated cells as control at days 0, 3, 7, 10 and 14.

The results showed cell viability decrease in time dependent manner. The lowest cell viability in erythroid progenitor cells treated with cytokines was found at day 14.

In healthy subjects, the cell viability of erythroid progenitor cells treated with 2, 20 and 40 ng/ml IL-1β at day 14 were 80.26, 75.17 and 77.79%, respectively. The cells viability of cells treated with 2, 20 and 40 ng/ml TNF-α were 79.51, 75.95 and 76.92%, respectively. The cell viability of cells treated with 2, 20 and 40 ng/ml IFN-γ were 81.22, 73.01 and 71.82%, respectively. The lowest cell viability was found in 40 ng/ml of IFN-γ (Figure 24).

In β-thalassemia/hemoglobin E, the cell viability of erythroid progenitor cells treated with 2, 20 and 40 ng/ml IL-1β at day 14 were 76.21, 68.21 and 70.32%, respectively. The cell viability of cells treated with 2, 20 and 40 ng/ml TNF-α were 69.82, 62.37 and 65.25%, respectively. The cell viability of cells treated with 2, 20 and 40 ng/ml IFN-γ were 72.43, 65.51 and 68.26%, respectively. The lowest cell viability was found in 20 ng/ml of TNF-α (Figure 25).

In anemia of chronic disease, the cell viability of erythroid progenitor cells treated with 2, 20 and 40 ng/ml IL-1β at day 14 were 78.02, 73.54 and 74.11%, respectively. The cell viability of cells treated with 2, 20 and 40 ng/ml TNF-α were 78.2, 75.67 and 74.12%, respectively. The cell viability of cells treated with 2, 20 and 40 ng/ml IFN-γ were 80.57, 73.26 and 73.69%, respectively. The lowest cell viability was found in 20 ng/ml of IFN-γ (Figure 26).

From the data indicated that cytokine treated cells had lower cell viability than untreated cells (control). Cell viability of erythroid progenitor cells from healthy subjects higher than anemia of chronic disease and β-thalassemia/HbE, respectively.

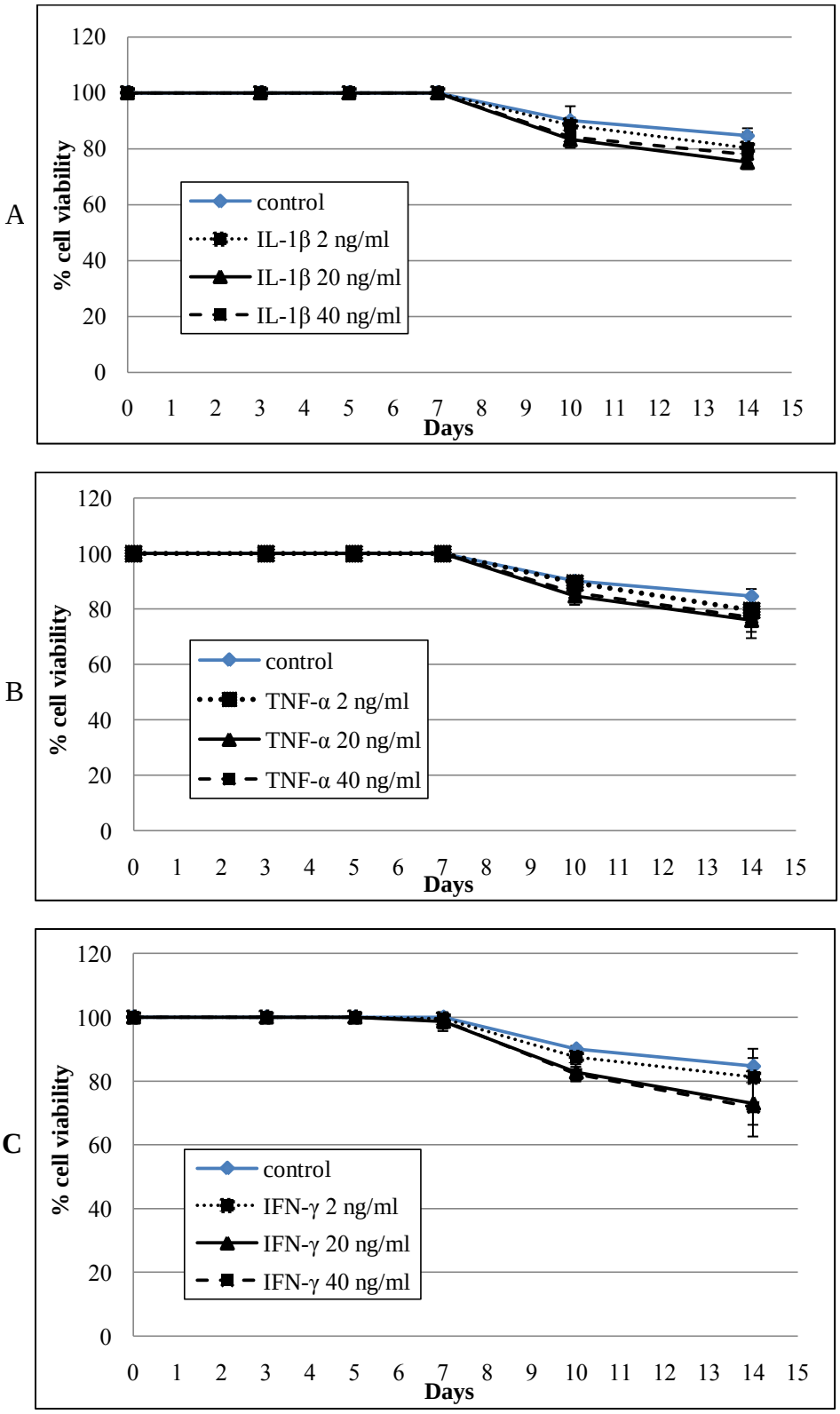


Figure 24. Mean cell viability of erythroid progenitor cells from healthy subjects. Cells treated with various concentrations and time of IL-1β (A), TNF-α (B) and IFN-γ (C).

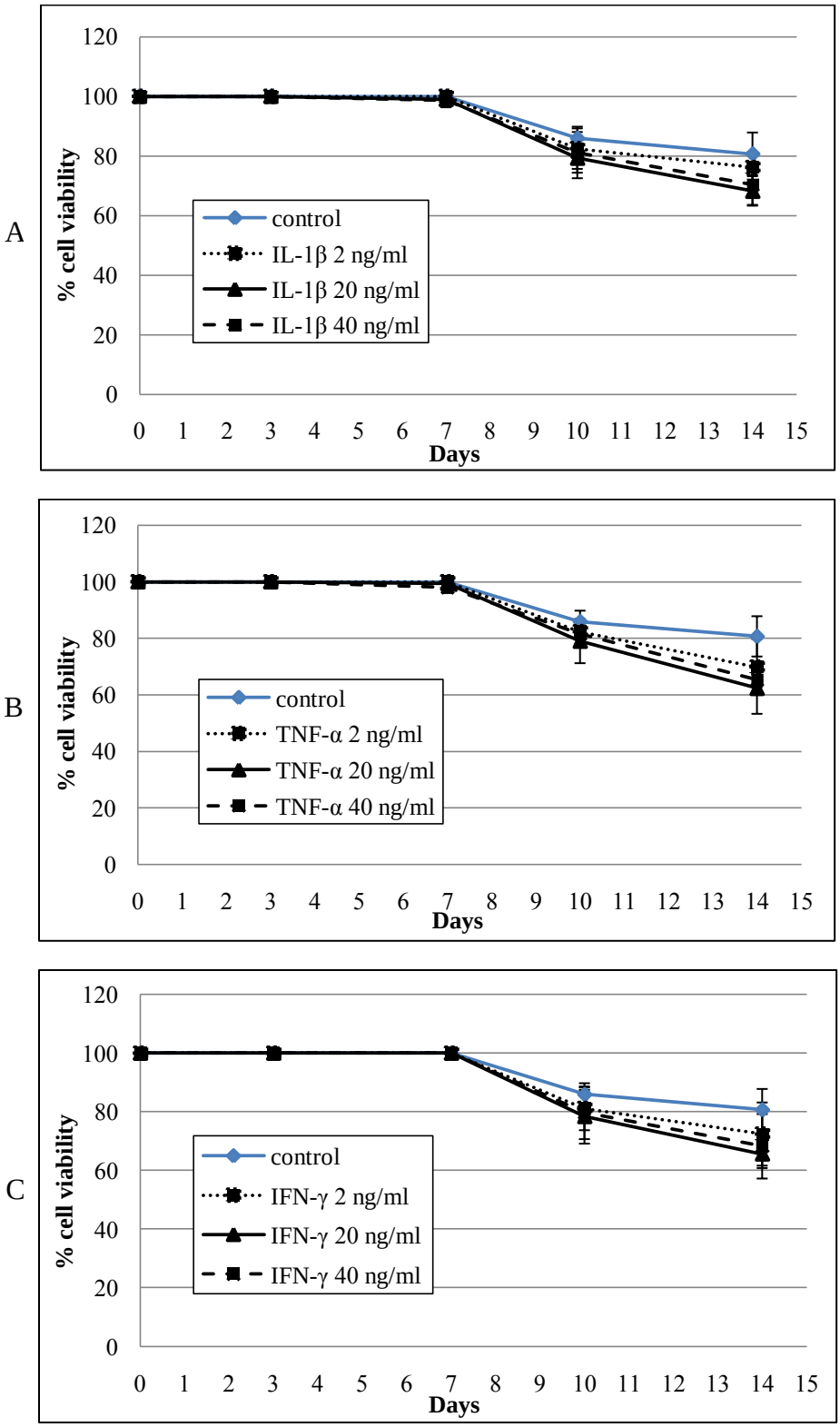


Figure 25. Mean cell viability of erythroid progenitor cells from β-thalassemia/HbE. Cells treated with various concentrations and time of IL-1β (A), TNF-α (B) and IFN-γ (C).

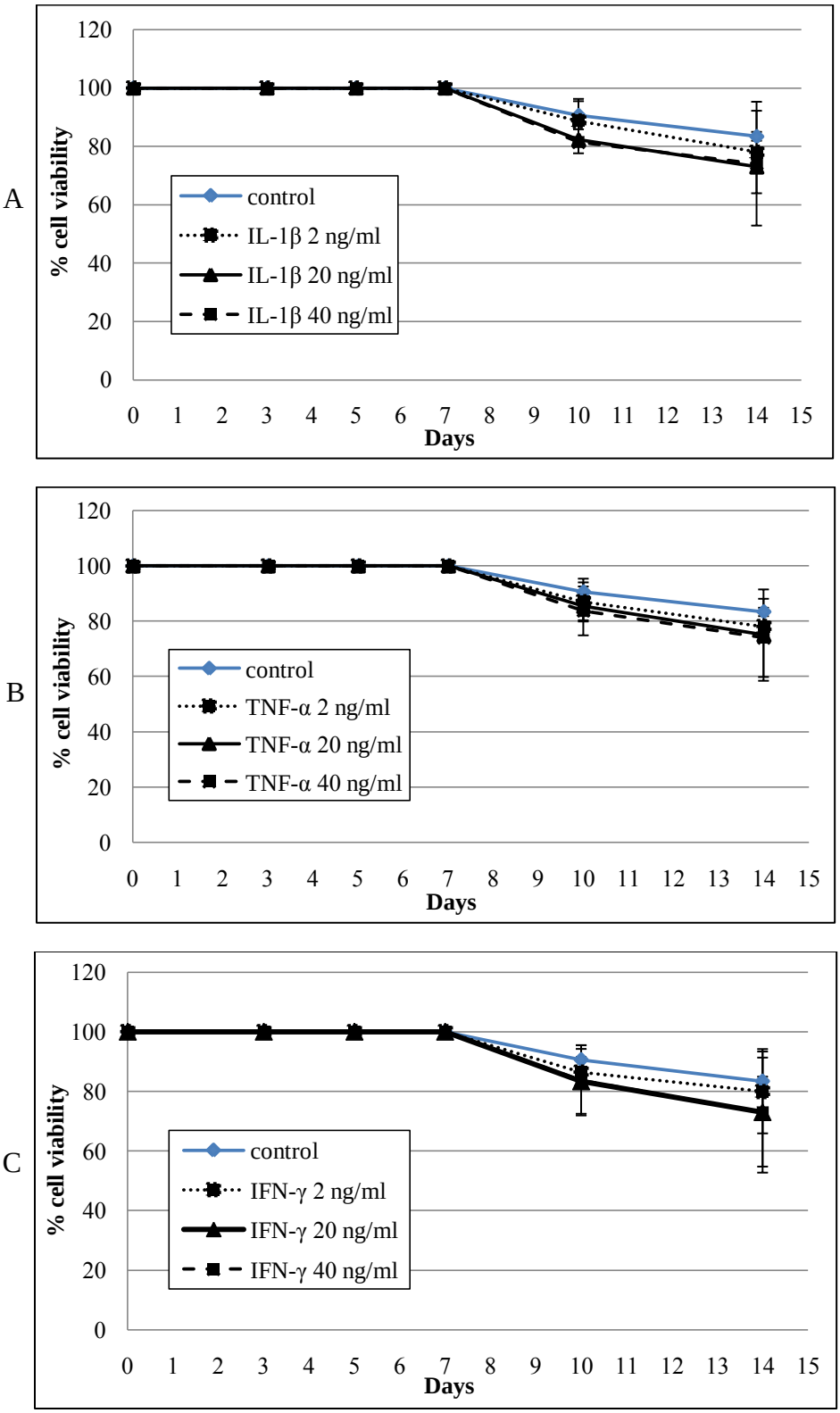


Figure 26. Mean cell viability of erythroid progenitor cells from anemia of chronic disease. Cells treated with various concentrations and time of IL-1β (A), TNF-α (B) and IFN-γ (C).

5.6 Effect of cytokines on cell apoptosis of erythroid progenitor cells

In order to investigate whether cytokines could induce cell apoptosis in erythroid progenitor cells. Cells treated with various concentrations of cytokines for 14 days and measured apoptosis by Annexin-V labeling FITC, then analysed by flow cytometry at days 7, 10 and 14 compared with control (untreated with cytokines). Annexin-V has been shown to interact strongly and specifically with phosphatidylserine (PS) residues and it can be used to detect apoptosis by targeting for the loss of plasma membrane. Glycophorin A-PE staining was also used simultaneously for erythroid marker.

In healthy subjects, the highest percentage of erythroid progenitor cells apoptosis was found in 40 ng/ml IFN-γ treated cells at day 7 (6.6±.64%), 20ng/ml TNF-α treated cells at day 10 (6.98±1.05%) and 20 ng/ml IFN-γ treated cells at day 14 (8.66±.69%) (Figure 27A).

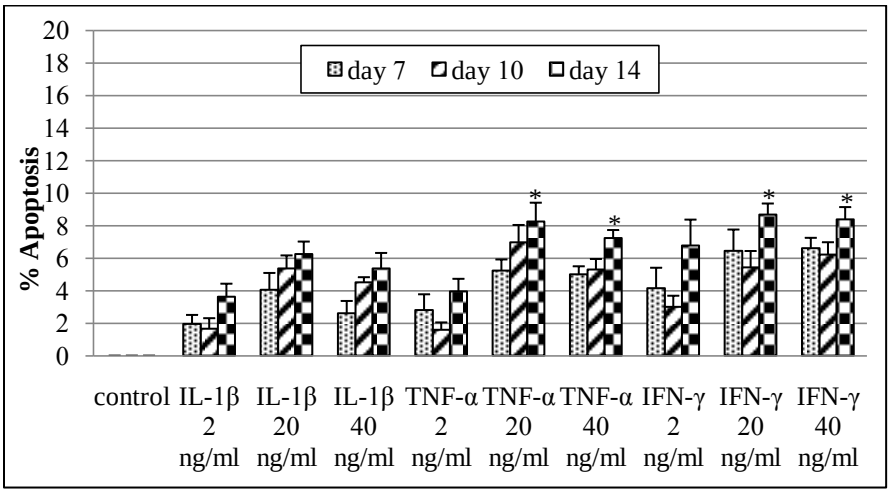
In β-thalassemia/hemoglobin E, the highest percentage of erythroid progenitor cells apoptosis was found in 40 ng/ml IFN-γ treated cells at day 7 (5.47±0.37%), 20 ng/ml IFN-γ treated cell at day 10 (12.84±1.24%) and 20 ng/ml TNF-α treated cells at day 14 (18.67±1.24%) (Figure 27B).

In anemia of chronic disease, the highest percentage of erythroid progenitor cells apoptosis was found in 40 ng/ml IFN-γ treated cells at day 7 (4.02±0.24%), 40 ng/ml IL-1β treated cells at day 10 (6.85±0.25%) and 20 ng/ml IFN-γ treated cells at day 14 (12.15±1.87%) (Figure 27C).

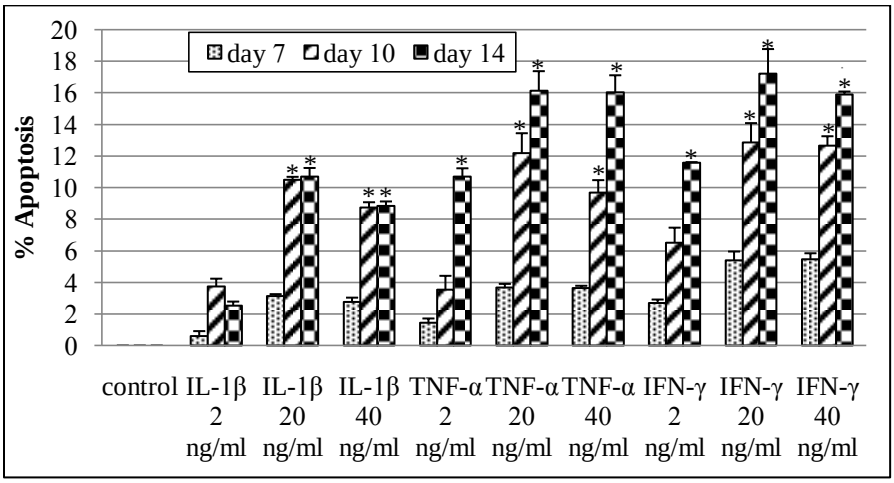
The results showed apoptosis was increased in time dependent manner. β-thalassemia/HbE had higher apoptosis than anemia of chronic disease and healthy subjects. The percentage of cells apoptosis was significantly higher ($P<0.05$) in cells treated with cytokines compared with control obvious in β-thalassemia/HbE. The highest percentage of erythroid progenitor cells apoptosis was found in 20 ng/ml TNF-α at day 14 (Figure 28).

From the results of total cell count, cell viability and cell apoptosis, the suitable concentrations of cytokines 20 ng/ml IL-1β, TNF-α and IFN-γ were used to examine the effect of cytokines on nitric oxide production and iNOS mRNA expression in next experiment.

A



B



C

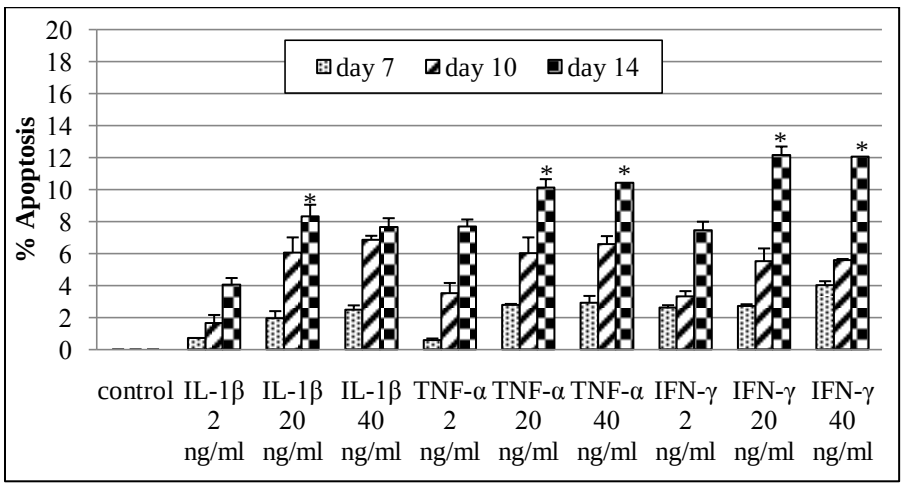


Figure 27. Percentage of erythroid progenitor cells apoptosis from healthy subjects (A), β-thalassemia/HbE (B) and anemia of chronic disease (C) treated with various concentrations of IL-1β, TNF-α and IFN-γ at day 7, 10 and 14.
(*, P<0.05 compared with control group).

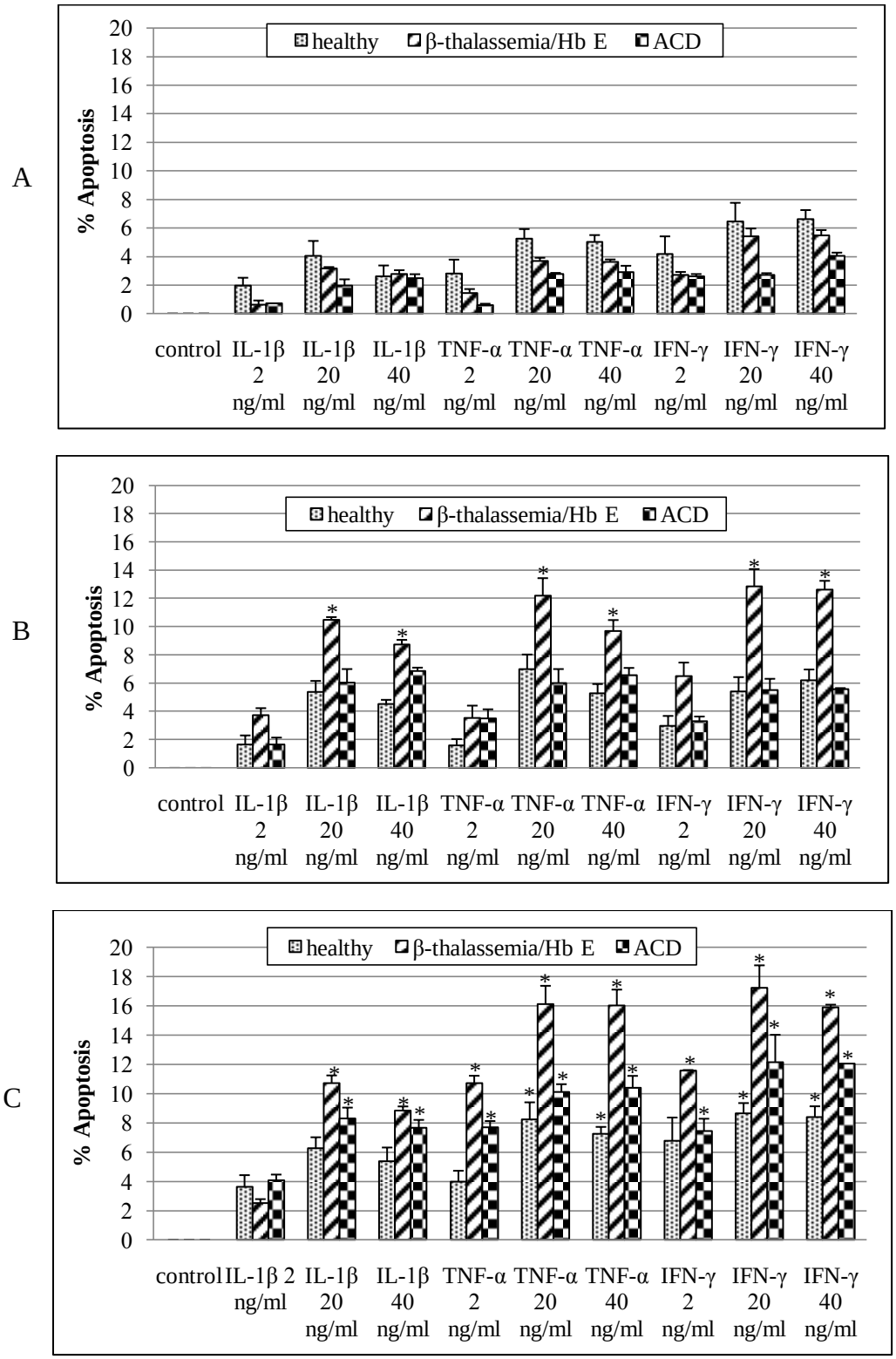


Figure 28. Percentage of erythroid progenitor cells apoptosis from healthy subjects, β-thalassemia/HbE and anemia of chronic disease treated with various concentrations of IL-1β, TNF-α and IFN-γ at days 7 (A), 10 (B) and 14 (C).
(*,P<0.05 compared with control group).

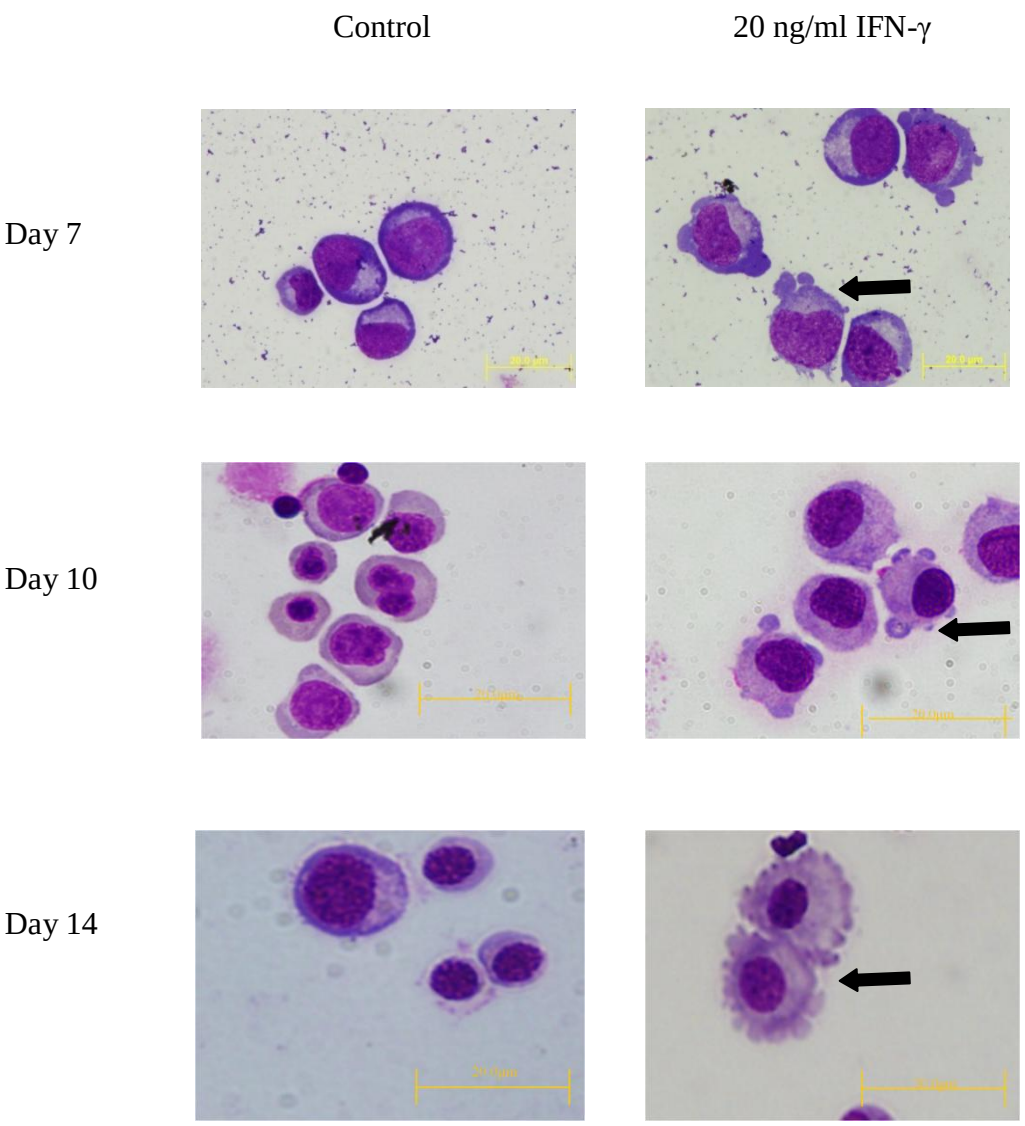


Figure 29. Wright-Giemsa staining of erythroid progenitor cells from β-thalassemia/ HbE. Cells were treated with 20 ng/ml IFN-γ compared with control at days 7, 10 and 14. Cell were then stained and observed apoptosis feature under light microscope.
⬅ Indicate apoptotic cell.

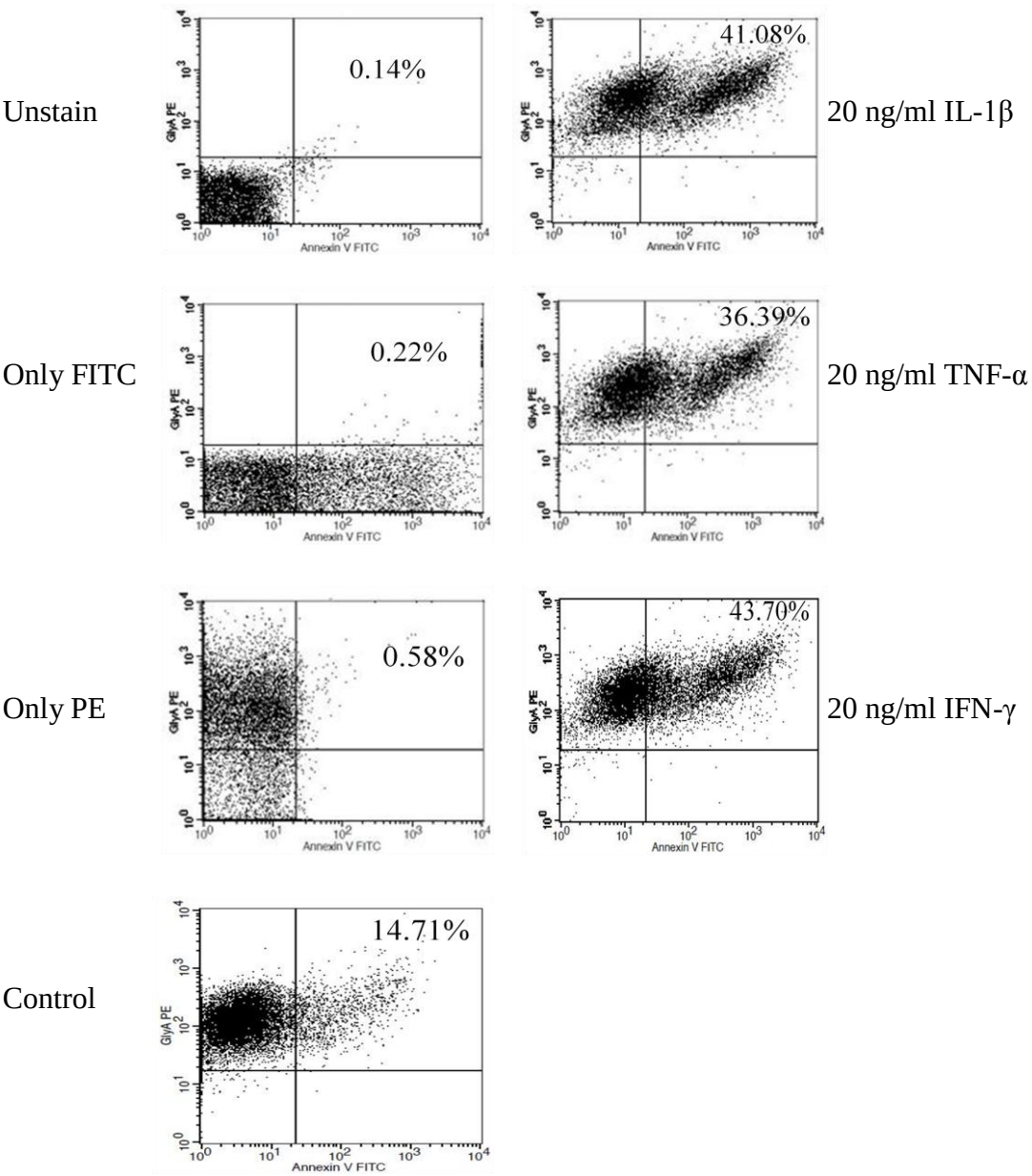


Figure 30. Flow cytometry analysis of Annexin V-FITC and GlycophorinA-PE staining of erythroid progenitor cell from β-thalassemia/HbE. Cells were treated with 20 ng/ml IL-1β, TNF-α and IFN-γ at day 14. The fluorescence intensity was analyzed by flow cytometry.

5.7 Effect of cytokines on iNOS mRNA expression in erythroid progenitor cells

To understand the mechanism of nitric oxide mediated cytokines induced apoptosis, we determined the effect of these cytokines on the iNOS mRNA expression in erythroid progenitor cells. Erythroid progenitor cells were cultured with cytokines and iNOS mRNA expression was determined by using real-time quantitative RT-PCR assay.

In healthy subjects, iNOS mRNA was not statistic significant increased after treatment with cytokines (P>0.05). The highest iNOS mRNA expression was found in 20 ng/ml IFN-γ at day 14 up to 4 fold increases (Figure 31A).

In β-thalassemia/hemoglobin E, cells cultured with cytokines showed statistic significant increased of iNOS mRNA compared with control (P<0.05*)*. The highest iNOS mRNA expression was found in 20 ng/ml IFN-γ treated cells at day 14 up to 20 fold increases (Figure 31B).

In anemia of chronic disease, 20 ng/ml TNF-α was not statistic significantly increase iNOS mRNA expression, whereas 20 ng/ml IL-1β and IFN-γ showed statistic significant increased iNOS mRNA at day 14 (P<0.05). The highest iNOS mRNA expression was found in 20 ng/ml IFN-γ at day 14 up to 6 fold increases (figure 31C).

The results showed that cytokine treated cells did not significantly increase iNOS mRNA expression in healthy subjects (P>0.05). In contrary, at day 10 and 14 after erythroid progenitor cells of β-thalassemia/hemoglobin E treated with cytokines were upregulated iNOS mRNA expression compared with control. In anemia of chronic disease showed statistic significant increased in cell treated cytokines only at day 14 (P<0.05). In addition, the highest iNOS mRNA expression shown in 20 ng/ml IFN-γ treated cells from β-thalassemia/HbE up to 20 fold increases (figure 32). Its implied iNOS mRNA expression upregulated by cytokines treatment in patients group.

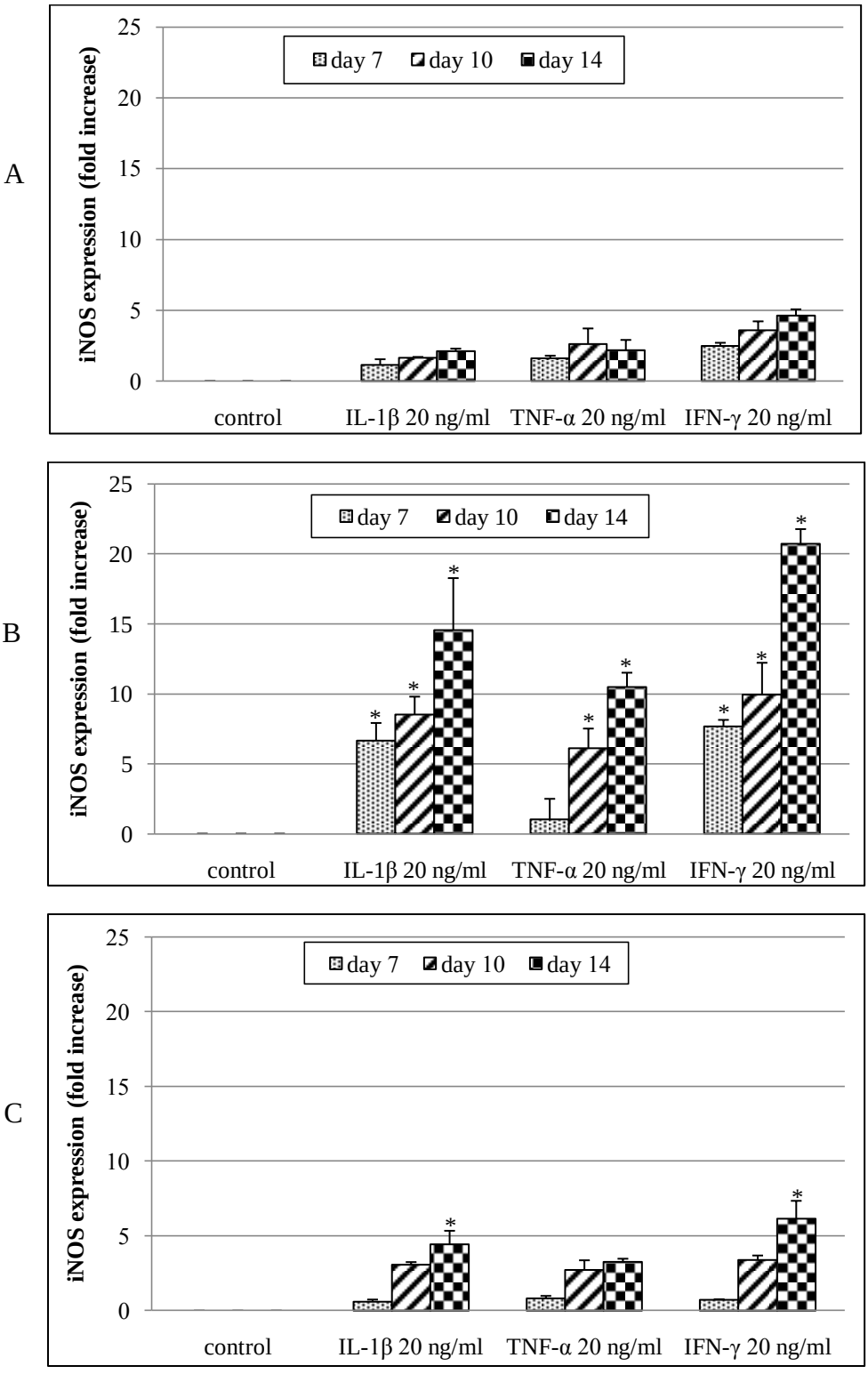


Figure 31. iNOS mRNA expression in erythroid progenitor cells from heaithy subjects (A), β-thalassemia/HbE (B) and anemia of chronic disease (C). Cells were treated with cytokine for 7, 10 and 14 days. iNOS mRNA expression was normalized to beta-2-microglobin (β_2M) housekeeping gene and analyzed by Real-time PCR.
(*,*P*<0.05 compared with control group)

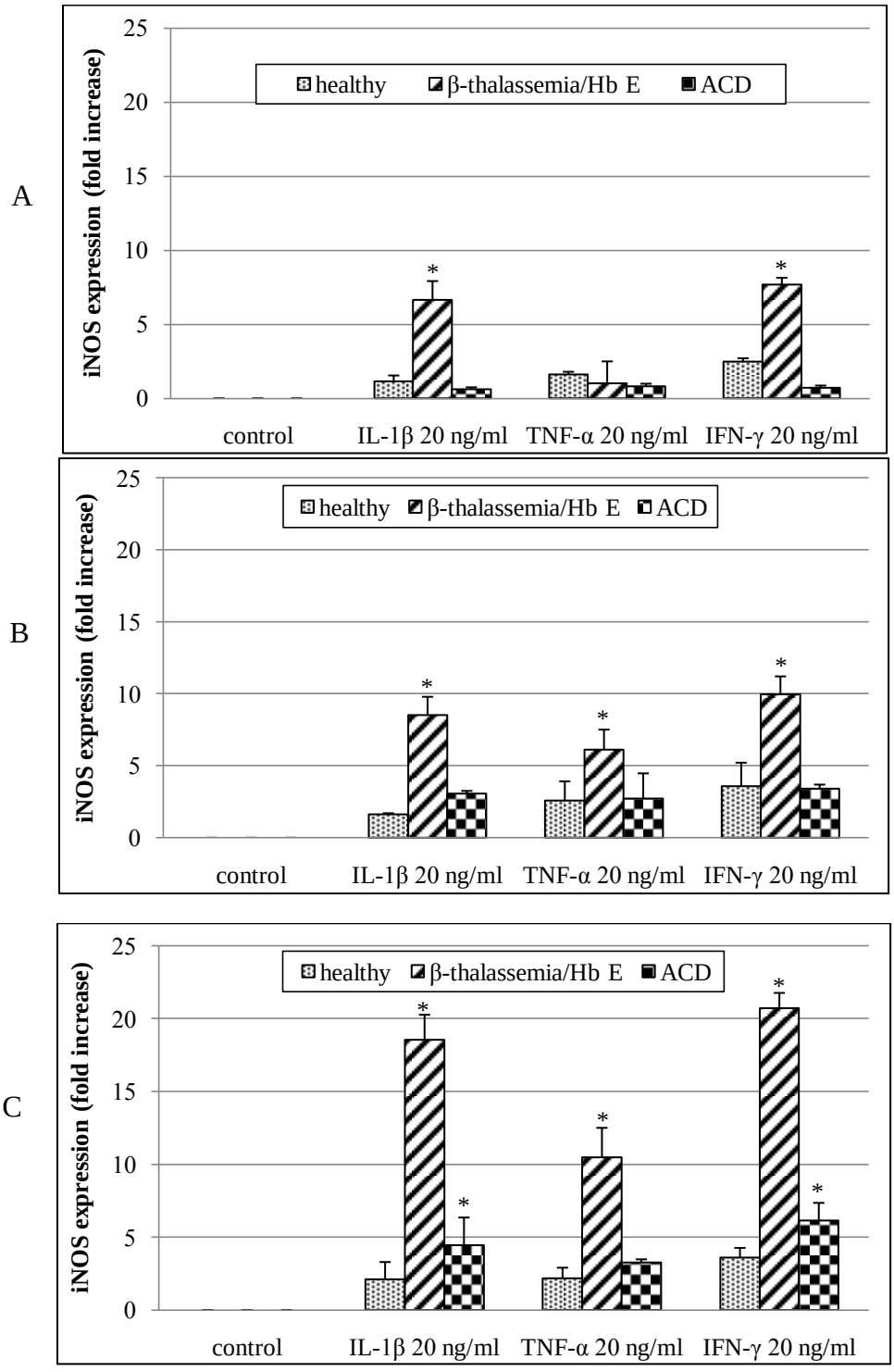


Figure 32. iNOS mRNA expression in erythroid progenitor cells from healthy subjects, β-thalassemia/HbE and anemia of chronic disease at days 7 (A), 10 (B) and 14 (C). iNOS mRNA expression was normalized to beta-2-microglobin (β_2M) housekeeping gene and analyzed by Real-time PCR.
(*,P<0.05 compared with control group)

5.8 Effect of cytokines on nitric oxide production

To understand the mechanism of cytokines, IL-1β, TNF-α and IFN-γ resulting in nitric oxide production mediated apoptosis. Supernatants from erythroid progenitor cells treated with cytokines were sampled and quantified the level of nitric oxide. Because of nitric oxide has a very short half-life time and unstable, so it is difficult to detect nitric oxide directly. Therefore, nitrite is stable end product of nitric oxide was determined by using Griess reagent. Results were compared against a $NaNo_2$ standard curve, and nitric oxide concentrations were calculated (Figure 33).

In healthy subjects, the levels of nitrite concentration decrease in time dependent manner. The highest levels of nitrite concentration was found in 20 ng/ml IFN-γ at day 7 compared with untreated cells as control (Figure 34A).

In β-thalassemia/HbE, the levels of nitrite concentration increase in time dependent manner. The highest levels of nitrite concentration was found in 20 ng/ml IFN-γ treated cell at day 10 (Figure 34B).

In anemia of chronic disease, the highest level of nitrite concentration was found in 20 ng/ml IFN-γ at day 14 (Figure 34C).

At day 7, the level of nitrite from erythroid progenitor cells treated with cytokines of healthy subjects higher than β-thalassemia/hemoglobin E and anemia of chronic disease (Figure 35A). Interestingly, at days 10 and 14, the level of nitrite concentration from β-thalassemia/hemoglobin E higher than healthy subjects and anemia of chronic disease in cells treated with cytokines (Figure 35B, C). The results showed cytokine treated cells induced nitric oxide production compared to untreated cells may involved in apoptotic processes of erythroid progenitor cells.

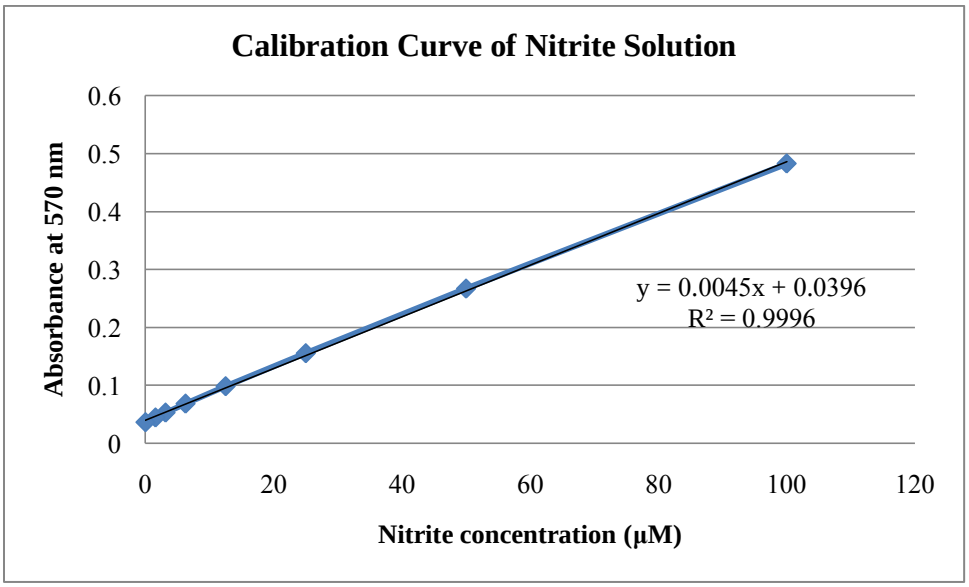


Figure 33. $NaNO_2$ standard curve (0-100 µM)

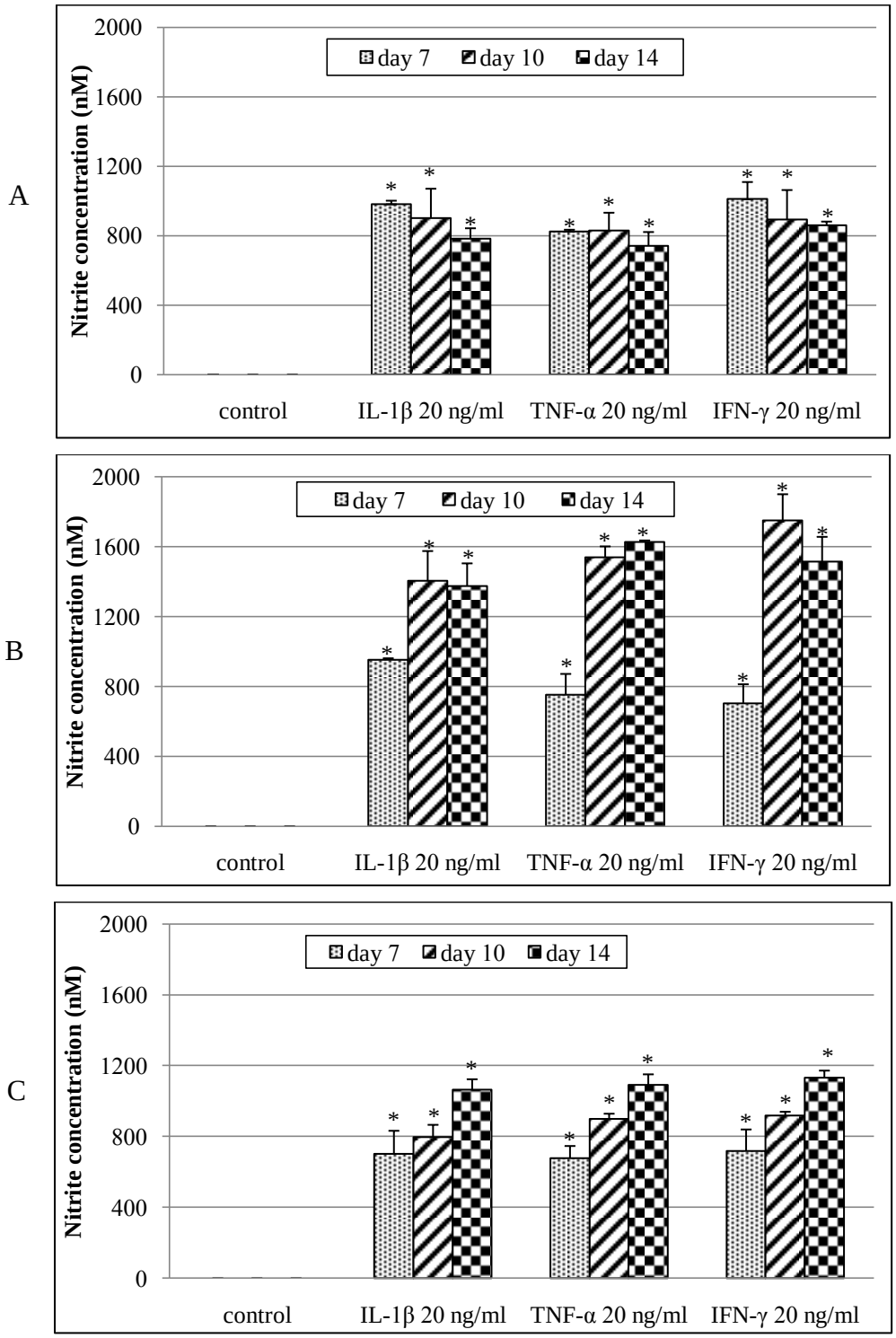


Figure 34. Nitrite concentration of erythroid progenitor cells from healthy subjects (A), β-thalassemia/HbE (B) and anemia of chronic disease (C). Cells were treated with IL-1β, TNF-α and IFN-γ at days 7, 10 and 14. Cell culture supernatants were assayed for relative levels of nitrite (NO_2^-) by Griess assay. (*, P<0.05 compared with control group).

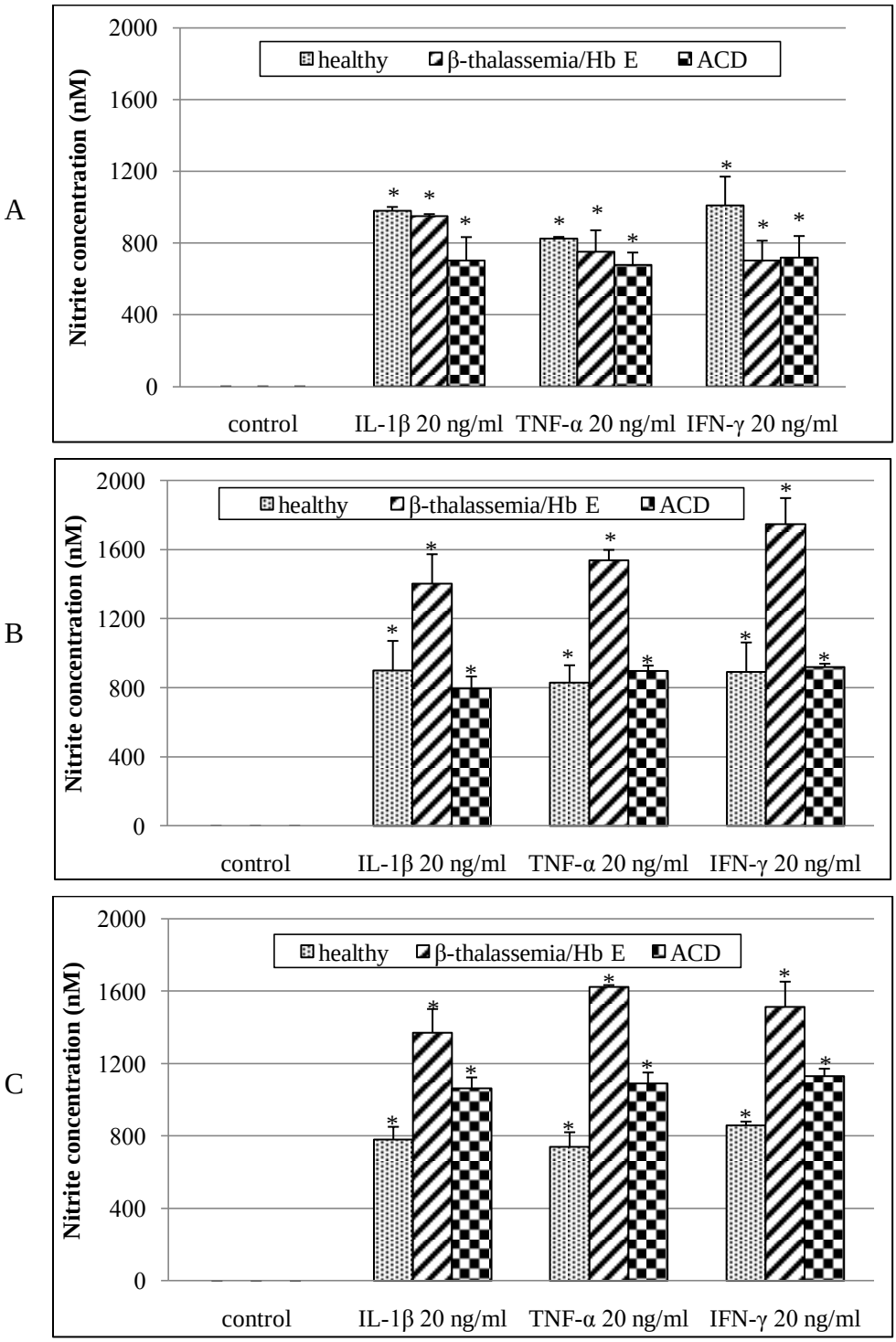


Figure 35. Nitrite concentration of erythroid progenitor cells from healthy subjects, β-thalassemia/HbE and anemia of chronic disease. Cells were treated with various concentration of IL-1β, TNF-α and IFN-γ at days 7 (A), 10 (B) and 14 (C). Cell culture supernatants were assayed for relative levels of nitrite (NO_2^-) by Griess assay. (*, P<0.05 compared with control group).

5.9 Effect of iNOS inhibitor on cytokine-induced apoptosis

To determine whether nitric oxide inhibitor could inhibit cytokines induced apoptosis. Erythroid progenitor cells were treated with 1 ng/ml of S-methyisothiourea Sulfate (SMT), a selective iNOS inhibitor, after three cytokines were added and cultured for 7, 10 and 14 days. Annexin V-FITC and glycophorin A-PE positive cell was assayed for cell apoptosis.

In healthy subjects, percentage of cell apoptosis from erythroid progenitor cells treated with 20 ng/ml IFN-γ incubated with SMT at day 7 showed statistic significant decrease percentage of cell apoptosis compared with cytokine treated cell without SMT about 4.18% (P<0.05) (Figure 36A). At day 10, percentage of cell apoptosis from erythroid progenitor cells treated with 20 ng/ml TNF-α and 20ng/ml IFN-γ incubated with SMT showed statistic significant decrease percentage of cell apoptosis compared with cytokine treated cells without SMT about 3.94 and 3.57%, respectively (P<0.05) (Figure 36B). At day 14, percentage of cell apoptosis from erythroid progenitor cells treated with 20 ng/ml IL-1β, 20 ng/ml TNF-α and 20ng/ml IFN-γ incubated with SMT showed statistic significant decrease percentage of cell apoptosis compared with cytokine treated cells without SMT about 4.99, 5.9 and 7.11%, respectively (P<0.05) (Figure 36C).

In β-thalassemia/hemoglobin E, percentage of cell apoptosis from erythroid progenitor cells treated with 20 ng/ml IFN-γ incubated with SMT at day 7 showed statistic significant decrease percentage of cell apoptosis compared with cytokine treated cells without SMT about 3.86% (P<0.05) (Figure 37A). At day 10, percentage of cell apoptosis from erythroid progenitor cells treated with 20 ng/ml IL-1β, 20 ng/ml TNF-α and 20ng/ml IFN-γ incubated with SMT showed statistic significant decrease percentage of cell apoptosis compared with cytokine treated cells without SMT about 6.99, 9.71 and 8.57%, respectively (P<0.05) (Figure 37B). At days 14, percentage of cell apoptosis from erythroid progenitor cells treated with 20 ng/ml IL-1β, 20 ng/ml TNF-α and 20ng/ml IFN-γ incubated with SMT showed statistic significant decrease percentage of cell apoptosis compared with cytokine treated cells without SMT about 6.87, 10.87 and 11.66%, respectively (P<0.05) (Figure 37C).

In anemia of chronic disease, percentage of cell apoptosis from erythroid progenitor cells treated with 20 ng/ml IFN-γ incubated with SMT at day 10 showed statistic significant decrease percentage of cell apoptosis compared with cytokine treated cells without SMT about 3.33% ($P<0.05$) (Figure 38B). At day 14, cell treated with 20 ng/ml IL-1β, 20 ng/ml TNF-α, 20ng/ml IFN-γ incubated with SMT showed statistic significant decrease percentage of cell apoptosis compared with cytokine treated cells without SMT about 4.42, 6.57 and 8.51%, respectively ($P<0.05$) (Figure 38C).

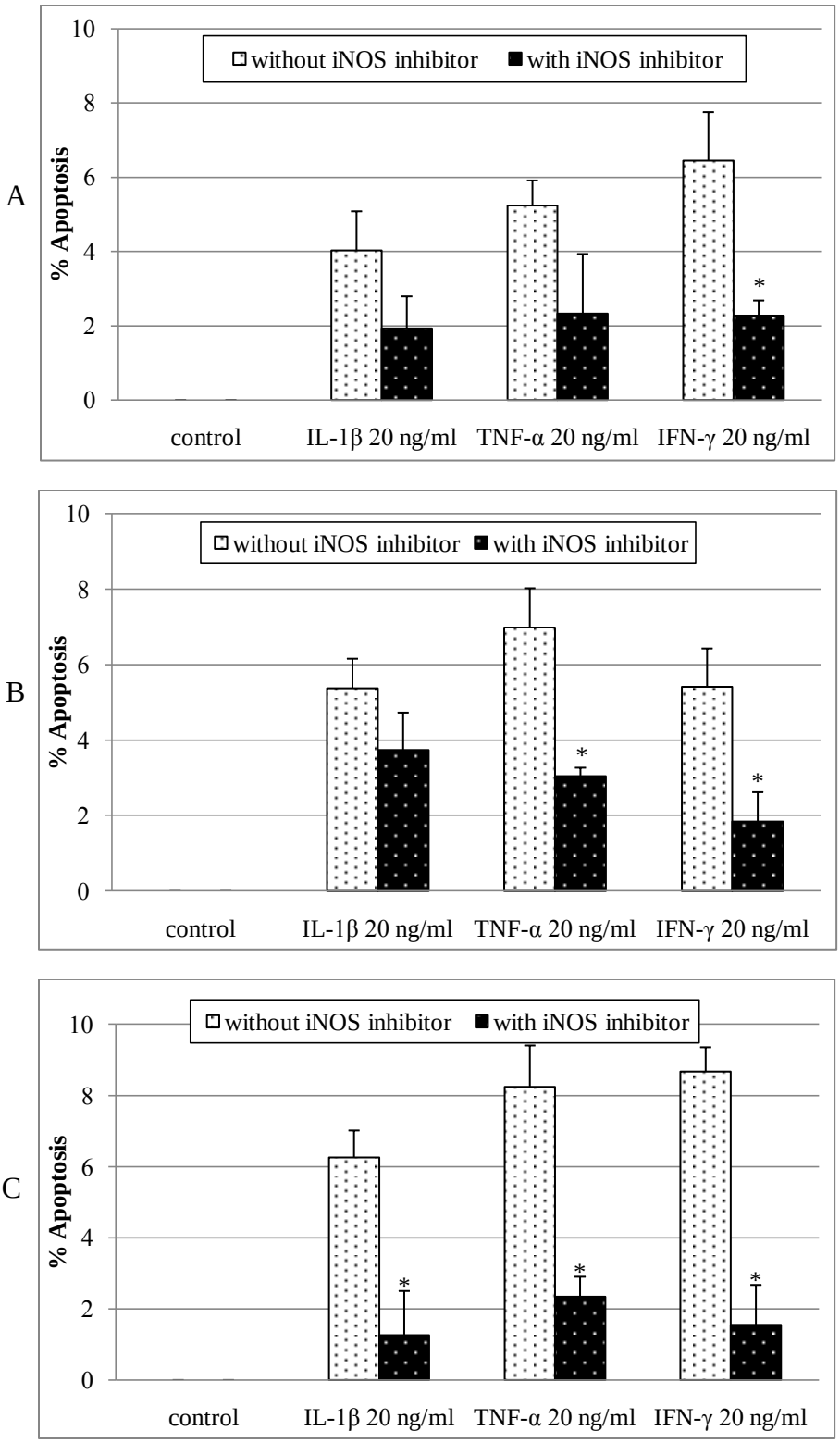


Figure 36. Percentage of cell apoptosis from healthy subjects. Cells were treated with IL-1β, TNF-α and IFN-γ and incubated with iNOS inhibitor at days 7 (A), 10 (B) and 14 (C). (*,P<0.05 compared with without iNOS inhibitor)

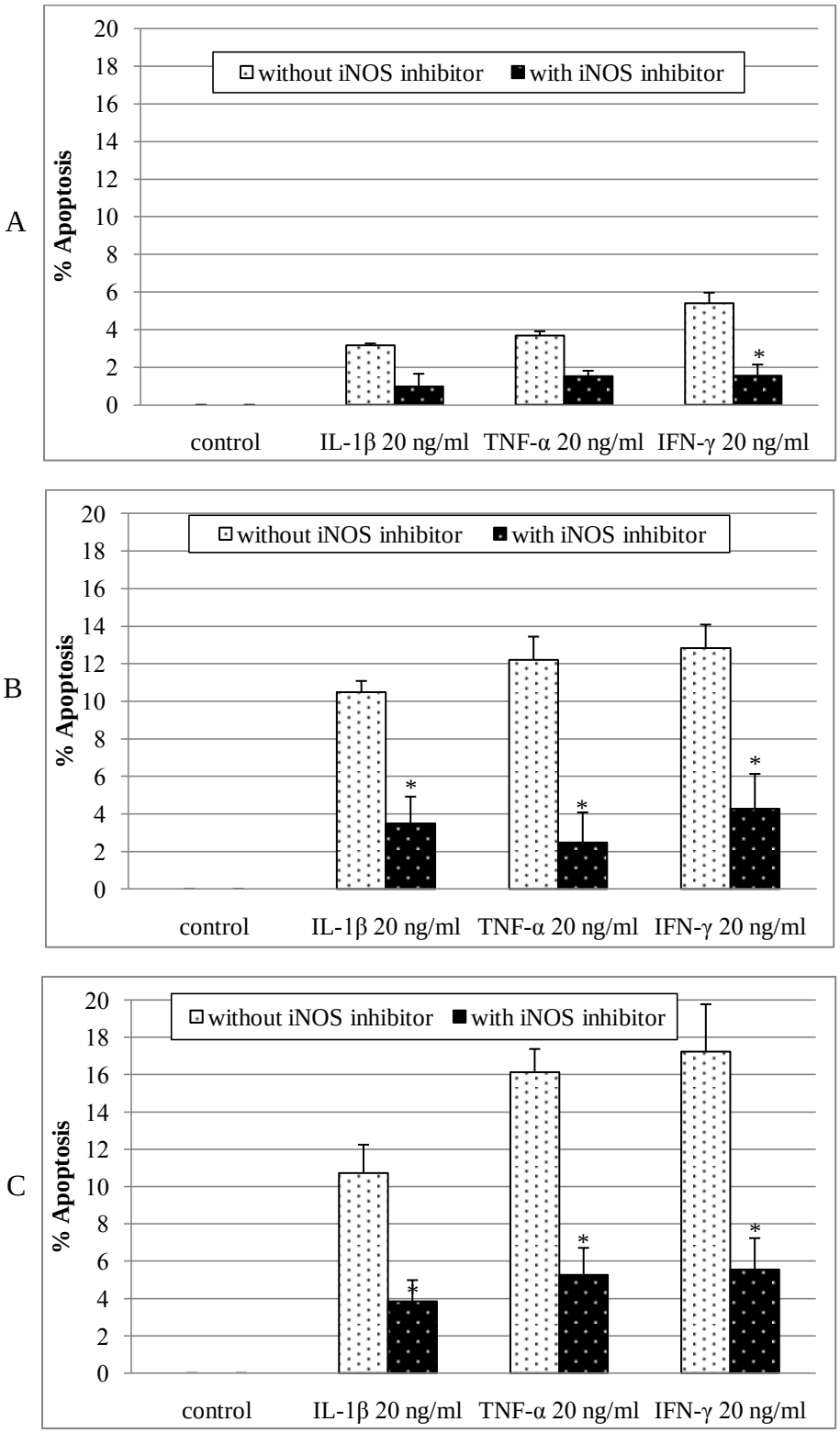


Figure 37. Percentage of cell apoptosis from β-thalassemia/HbE. Cells were treated with IL-1β, TNF-α and IFN-γ and incubated with iNOS inhibitor at days 7 (A), 10 (B) and 14 (C). (*,P<0.05 compared with without iNOS inhibitor)

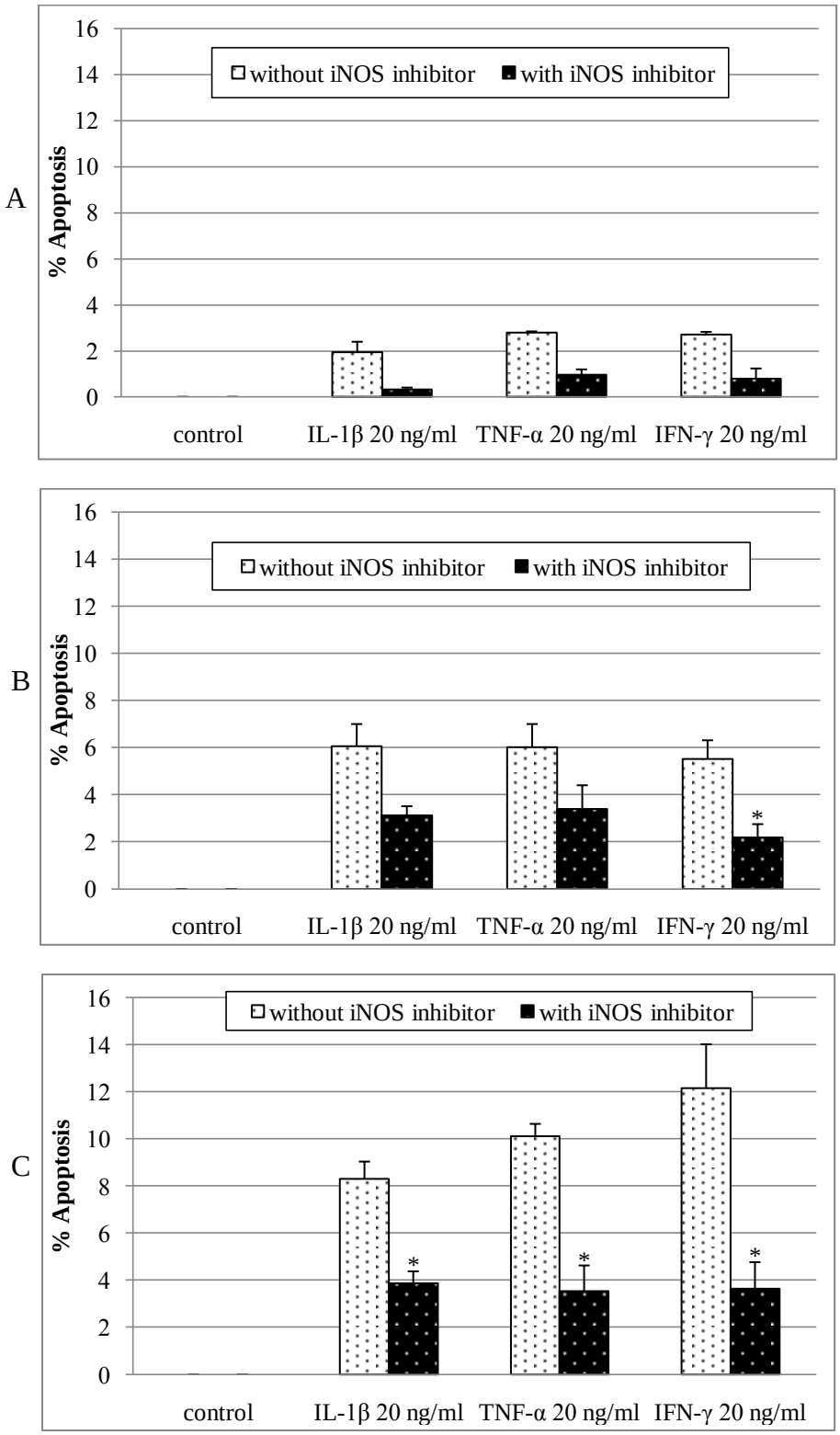


Figure 38. Percentage of cell apoptosis from anemia of chronic disease. Cells were treated with IL-1β, TNF-α and IFN-γ and incubated with iNOS inhibitor at days 7 (A), 10 (B) and 14 (C). (*,P<0.05 compared with without iNOS inhibitor)

5.10 Effect of iNOS inhibitor on cytokine-induced iNOS mRNA expression

To confirm about NO mediated cytokines induced apoptosis by iNOS expression. Erythroid progenitor cells were treated with selective inhibitor of iNOS, S-methyisothiourea Sulfate (SMT), after three cytokines were added and cultured for 7, 10 and 14 days. iNOS mRNA expression was determined using real-time quantitative RT-PCR assay.

In healthy subjects, erythroid progenitor cells treated with 20 ng/ml IFN-γ incubated with SMT at day 7 showed statistic significant decrease of iNOS mRNA expression compared with 20 ng/ml IFN-γ treated cell without SMT about 1.57 fold (P<0.05) (Figure 39A). At day 10, cells treated with 20 ng/ml TNF-α and 20 ng/ml IFN-γ incubated with SMT showed statistic significant decrease of iNOS mRNA expression compared with cytokine treated cells without SMT about 1.71 and 2.48 fold (P<0.05) (Figure 39B). At day 14, cells treated with 20 ng/ml IFN-γ incubated with SMT showed statistic significant decrease of iNOS mRNA expression compared with cytokine treated cells without SMT about 3.41 fold (P<0.05) (Figure 39C).

In β-thalassemia/hemoglobin E, erythroid progenitor cells treated with 20 ng/ml IL-1β, TNF-α and IFN-γ incubated with SMT at day 10 showed statistic significant decrease of iNOS mRNA expression compared with cytokine treated cells without SMT about 6.93, 4.5 and 8.64 fold, respectively (P<0.05) (Figure 40B). At day 14, cell treated with cytokines, 20 ng/ml IL-1β, TNF-α and IFN-γ incubated with SMT showed statistic significant decrease of iNOS mRNA expression compared with cytokine treated cells without SMT about 10.04, 5.12 and 15.22 fold, respectively (P<0.05) (Figure 40C).

In anemia of chronic disease, erythroid progenitor cells treated with 20 ng/ml IL-1β and IFN-γ incubated with SMT at day 10 showed statistic significant decrease of iNOS mRNA expression compared with cytokine treated cells without SMT about 2.24 and 2.47 fold, respectively (P<0.05) (Figure 41B). At day 14, erythroid progenitor cells treated with 20 ng/ml IL-1β, TNF-α and IFN-γ incubated with SMT showed statistic significant decrease of iNOS mRNA expression compared with cytokine treated cells without SMT about 2.67, 1.76 and 3.81 fold, respectively (P<0.05) (Figure 41C).

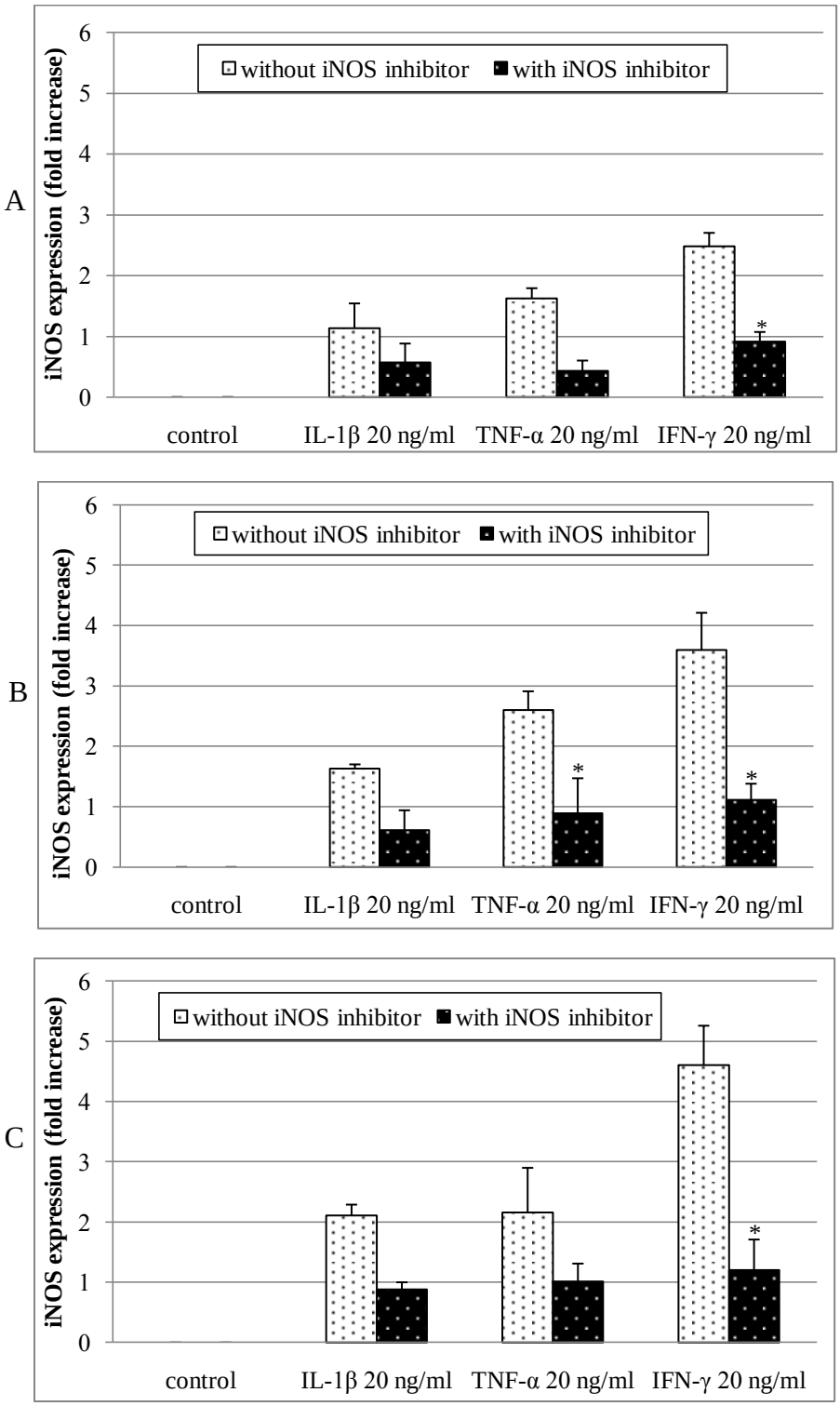


Figure 39. iNOS mRNA expression of erythroid progenitor cell from healthy subjects. Cells were treated with IL-1β, TNF-α and IFN-γ and incubated with iNOS inhibitor for 7 (A), 10 (B) and 14 (C) days. iNOS mRNA expression was normalized to beta-2-microglobulin (β_2M) housekeeping gene and analyzed by Real-time PCR. (*,P<0.05 compared with without iNOS inhibitor)

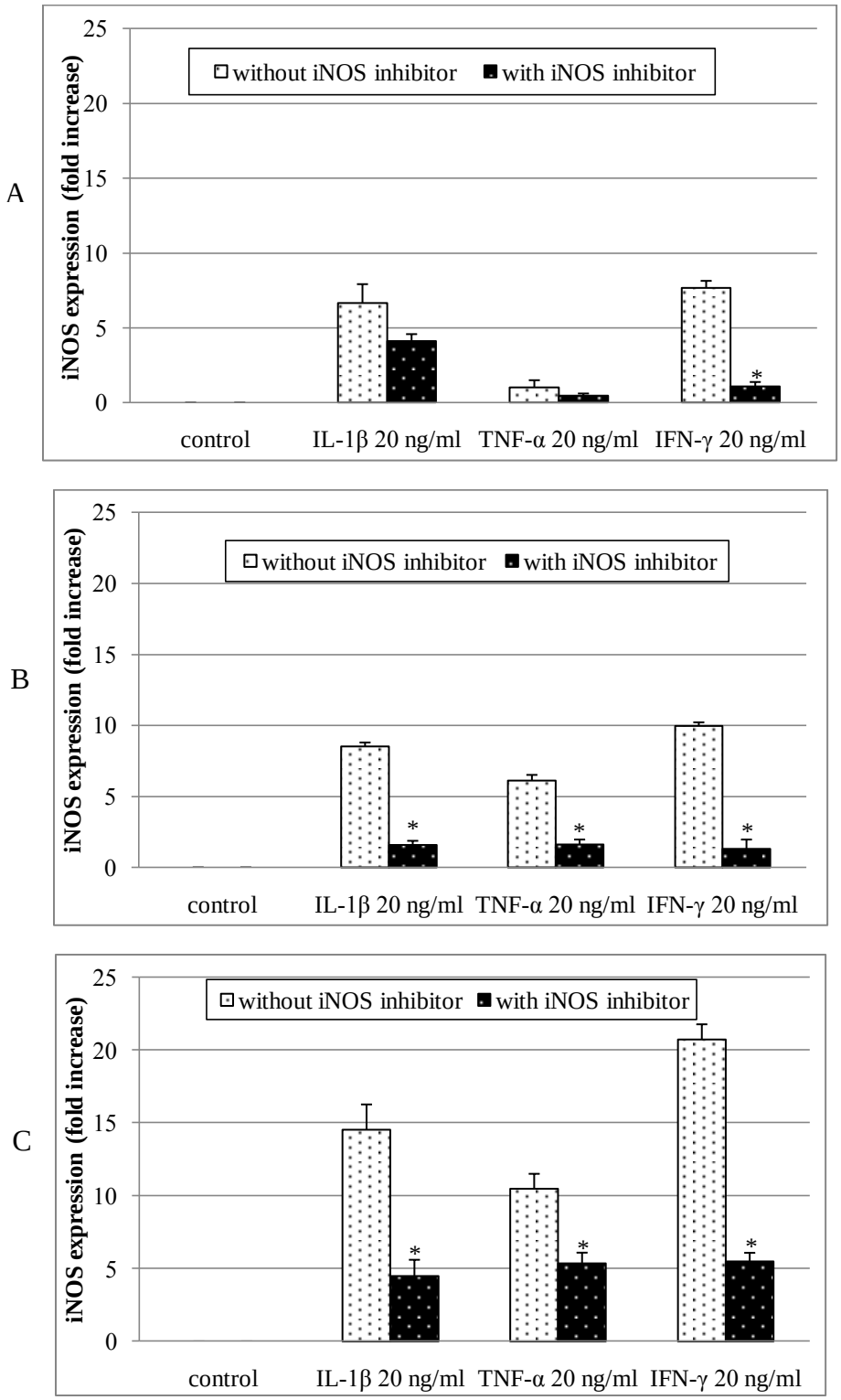


Figure 40. iNOS mRNA expression of erythroid progenitor cell from β-thalassemia/HbE. Cells were treated with IL-1β, TNF-α and IFN-γ and incubated with iNOS inhibitor for 7 (A), 10 (B) and 14 (C) days. iNOS mRNA expression was normalized to beta-2-microglobulin (β_2M) housekeeping gene and analyzed by Real-time PCR. (*,P<0.05 compared with without iNOS inhibitor)

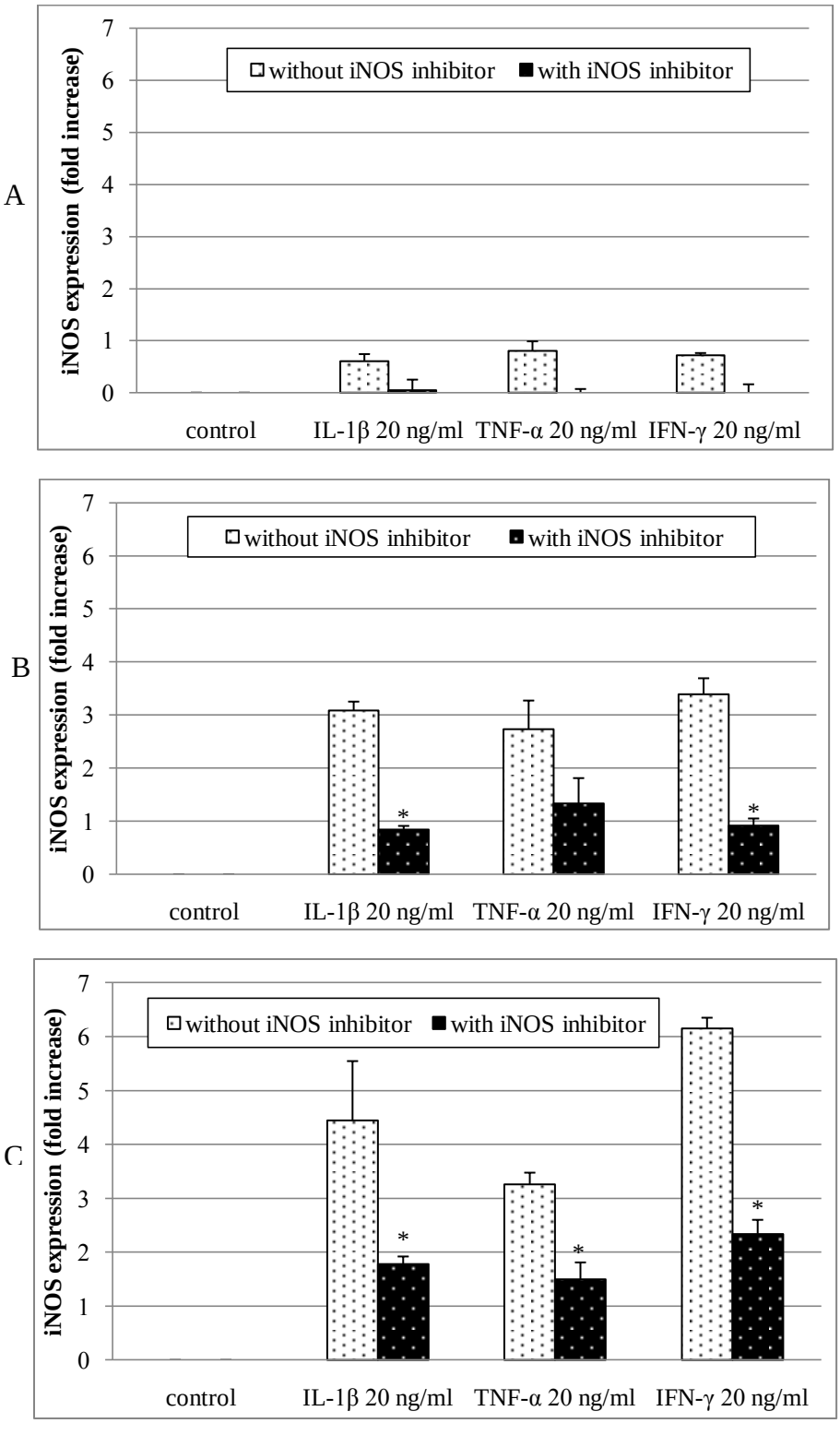


Figure 41. iNOS mRNA expression of erythroid progenitor cell from anemia of chronic disease. Cells were treated with IL-1β, TNF-α and IFN-γ and incubated with iNOS inhibitor for 7 (A), 10 (B) and 14 (C) days. iNOS mRNA expression was normalized to beta-2-microglobulin (β_2M) housekeeping gene and analyzed by Real-time PCR. (*,P<0.05 compared with without iNOS inhibitor)

5.11 Effect of iNOS inhibitor on cytokine-induced NO production

To determine whether NO is indeed the toxic mediator of cytokine-induced apoptosis. Erythroid progenitor cells were treated with cytokines with or without iNOS inhibitor, S-methyisothiourea Sulfate (SMT), for 7, 10 and 14 days. Nitrite (NO_2^-), NO end product, was determine by Griess method.

In healthy subjects, erythroid progenitor cells treated with 20 ng/ml IL-1β produced nitrite up to 980 nM. However, after incubation 620 ng/ml IFN-γ with SMT, nitrite production was reduced to 1020 nM (Figure 42A). At days 10 and 14, erythroid progenitor cells treated with cytokines incubated with SMT showed significant reduced the level of nitrite compared with cytokine treated cells without SMT (P<0.05) (Figure 42B, C).

In β-thalassemia/hemoglobin E, erythroid progenitor cells treated with 20 ng/ml IL-1β and 20 ng/ml TNF-α produced nitrite up to 950 and 750 nM, respectively. However, after incubation of cytokines with SMT, nitrite production was reduced to 320 and 290 nM, respectively (Figure 43A). At days 10 and 14, erythroid progenitor cells treated with cytokines incubated with SMT showed significant reduced the level of nitrite compared with cytokine treated cells without SMT (P<0.05) (Figure 43B, C).

In anemia of chronic disease, erythroid progenitor cells treated with cytokines incubated with SMT showed significant reduced the level of nitrite compared with cytokine treated cells without SMT (P<0.05) (Figure 44).

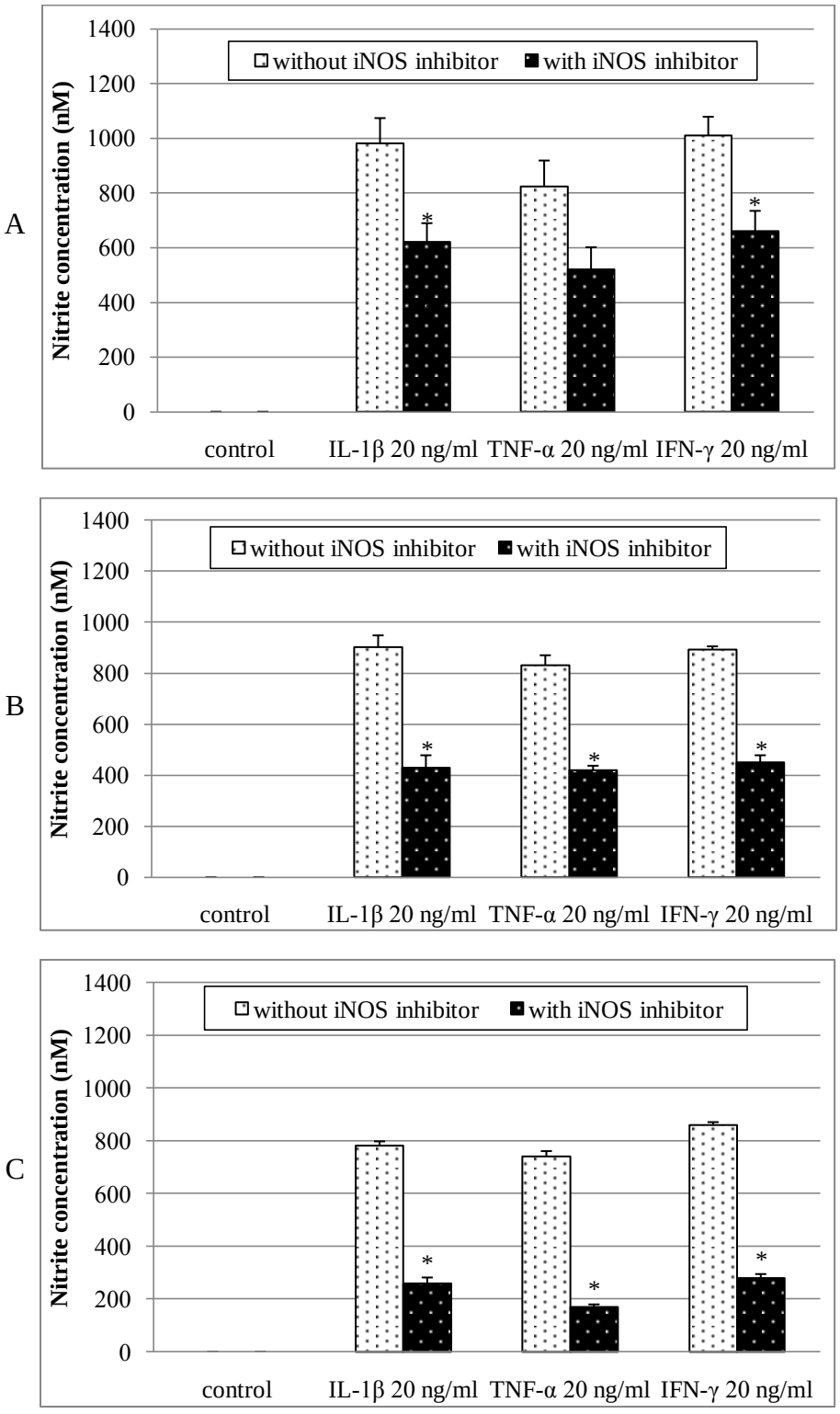


Figure 42. Nitrite concentration of erythroid progenitor cells from healthy subjects. Cells were treated with IL-1β, TNF-α and IFN-γ and incubated with iNOS inhibitor for 7 (A), 10 (B), 14 (C) days. Cell culture supernatants were assayed for relative levels of Nitrite (NO_2^-) by Griess assay. (*,P<0.05 compared with without iNOS inhibitor)

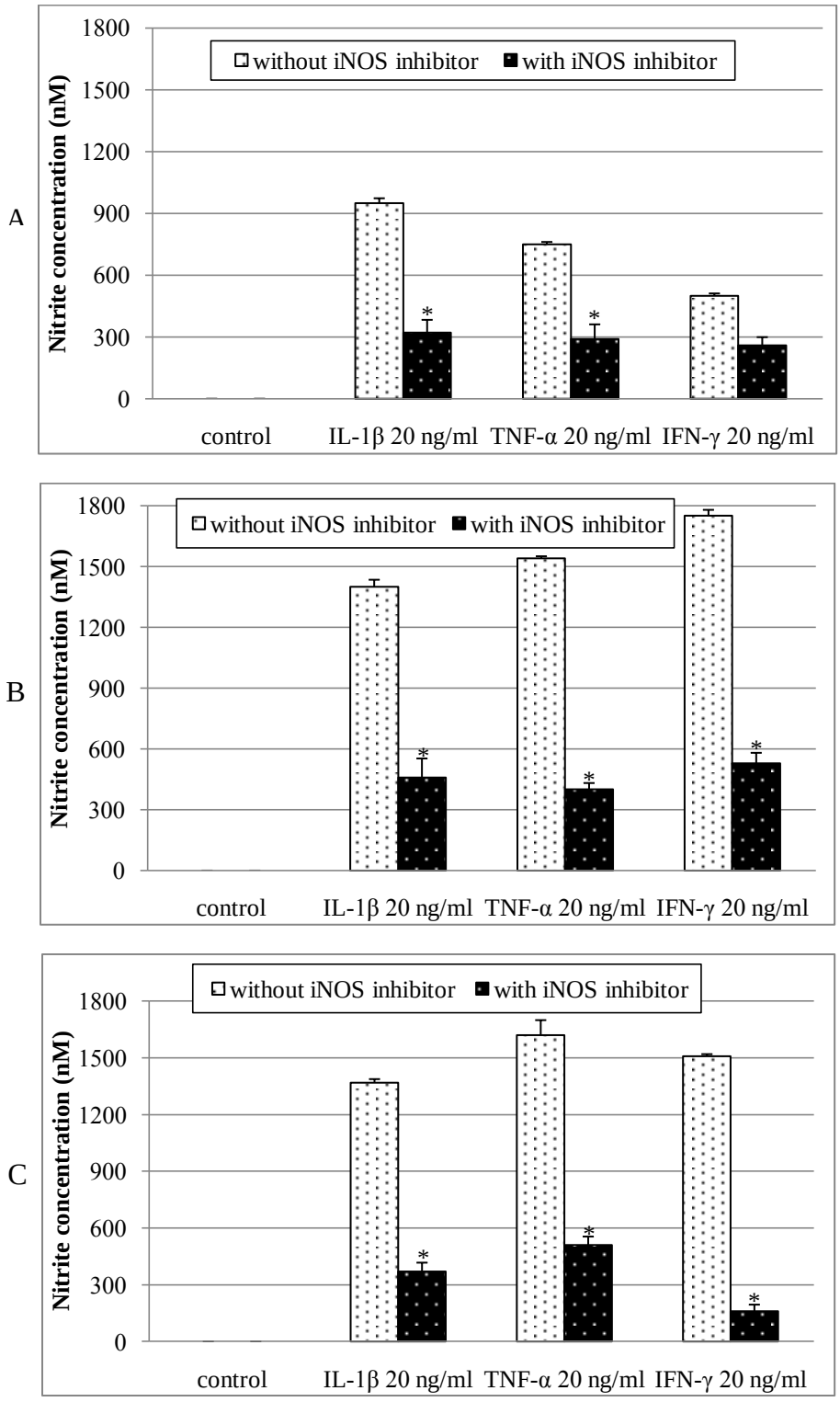


Figure 43. Nitrite concentration of erythroid progenitor cells from β-thalassemia/HbE. Cells were treated with IL-1β, TNF-α and IFN-γ and incubated with iNOS inhibitor for 7 (A), 10 (B), 14 (C) days. Cell culture supernatants were assayed for relative levels of Nitrite (NO_2^-) by Griess assay. (*,P<0.05 compared with without iNOS inhibitor)

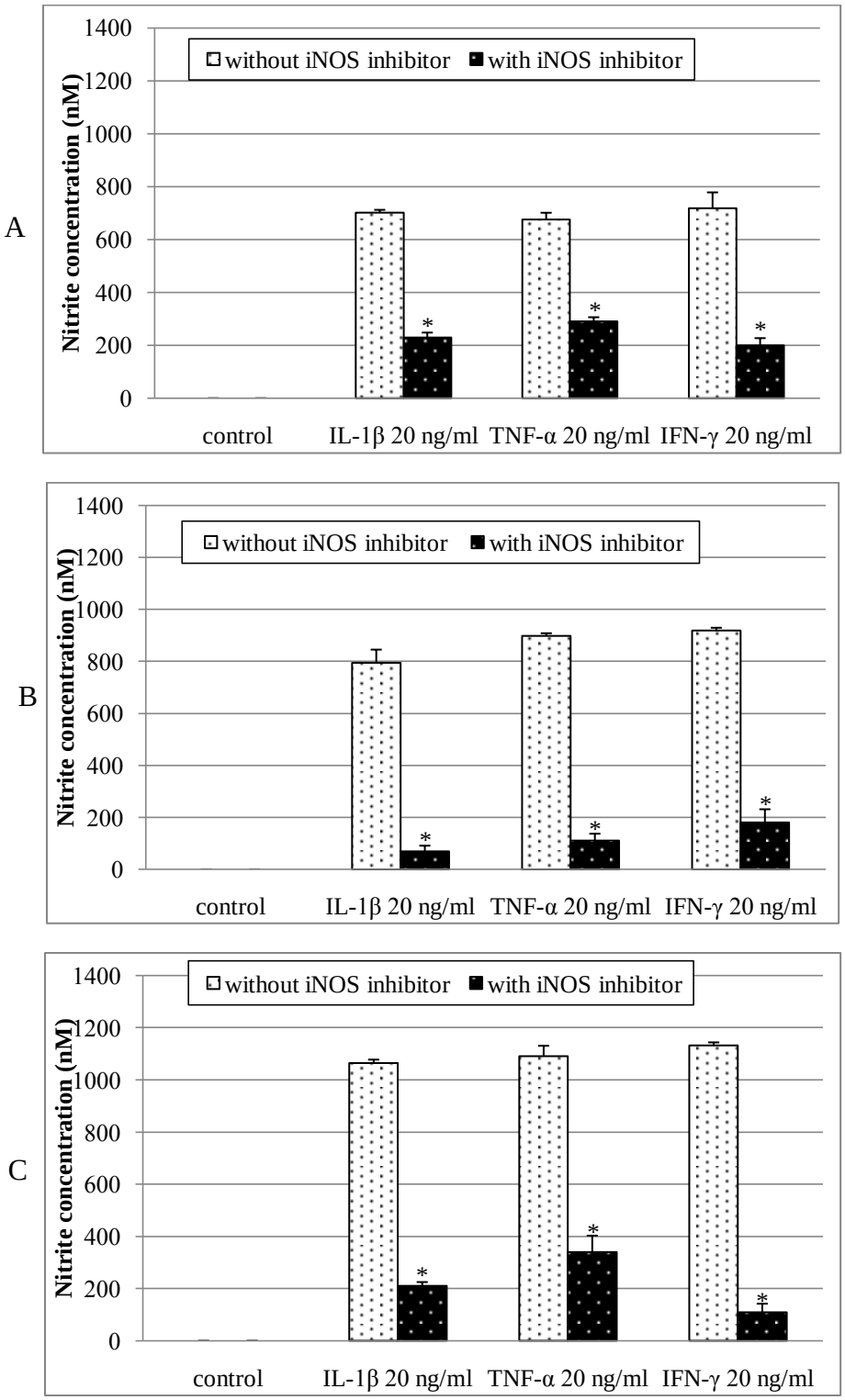


Figure 44. Nitrite concentration of erythroid progenitor cells from anemia of chronic disease. Cells were treated with IL-1β, TNF-α and IFN-γ and incubated with iNOS inhibitor for 7 (A), 10 (B), 14 (C) days. Cell culture supernatants were assayed for relative levels of Nitrite (NO_2^-) by Griess assay. (*,P<0.05 compared with without iNOS inhibitor)

CHAPTER VI
DISCUSSION

Erythropoiesis is a process of red blood cell formation or production. In humans, erythropoiesis occurs almost exclusively in the bone marrow. There are evidences supporting that the erythroid progenitor cells of thalassemic patients underwent apoptosis more than the healthy erythroid progenitor cells (14). However, the mechanisms of accelerated apoptosis in β-thalassemia/hemoglobin E was not been well documented. Thalassemia is the result of an excess of α or β chains, release of heme and heme iron. The iron overload found in β-thalassemia/hemoglobin E can lead to an iron interstinal hyperabsorption and an abnormal molecular iron form (non-transferrin bound: NTBI) accumulation. NTBI and iron are known to be involved in several chemical reactions that generate reactive species contributes to the formation of free radicals and increase apoptosis process (6). Furthermore, possibility cause of apoptosis may associate with microenvironment in the cell such as growth factors and cytokines. In recent research showed thalassemic patients had significantly increase level of IL-1β, TNF-α and IFN-γ (8).

Anemia of chronic disease (ACD) is the most frequent among hospitalized patients develops under chronic inflammatory disorder. Anemia of chronic diseases associated with anemia in difference pathology from β-thalassemia/hemoglobin E. Most case of anemia occurring in patients with rheumatoid arthritis (RA) are characterized as anemia of chronic disease (39). It is considered that genetic, environmental, and hormonal factors contribute to the development of RA, although its etiology and pathogenesis are still incompletely understood. Previous studies have suggested that various factors contribute to the pathogenesis of ACD. Shortened red cell survival, impaired reticuloendothelial iron release, impaired bone marrow response to anemia, and insufficient amount or altered activity of erythropoietin. Cytokine network has been studied in RA patients. Proinflammatory cytokines such as

tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) play a significant role in pathogenesis of RA (48).

Both β-thalassemia/hemoglobin E and anemia of chronic disease were disturbance cytokine system, which increase proinflammatory cytokine production. Then, cytokines may involved in pathological processes. Many cytokines involved in acute and chronic phase response an inhibitory activity on erythroid colony formation *in vitro*. The cytokines, IL-6, IL-1 that have a major inhibitory effect on red cell precursors in the bone marrow (13). Transforming growth factor beta (TGF-β) inhibits the earliest erythroid precursors and BFU-E both directly by inhibiting the activity of IL-3. The inhibitory effect of TNF-α on the growth of BFU-E and CFU-E is directly, at least partly mediated by the IFN-γ produced by bone marrow stromal cells (65). Likewise indirect is the inhibitory effect of IL-1 on CFU-E generation and is mediated by the IFN-γ produced by T cells (66). The cytokines that negatively regulate erythroid precursors act synergistically, as consistently reported for IL-1 and TNF-α. Circulating TNFα is elevated in RA (68) and IL-1β serum levels are significantly increased in RA with ACD as compared to RA without anemia (69). Moreover, it was also reported that in β-thalassemia/HbE showed increase level of cytokines include TNF-α, IL-1β and IFN-γ correlated with their clinical symptom, suggesting the possibility that cytokines might contribute to the inhibit erythropoietic activity.

This study evaluated effect of cytokines, IL-1β, TNF-α, and IFN-γ in various concentration on cell count, cell viability, and cell apoptosis. The concentration for this experiment applied from previous study. Umesh's thesis investigated role of IL-1 in apoptosis related to the ineffective erythropoiesis in β-thalassemia/HbE, he found the highest number of erythroid annexin V positive cell was noted in the culture treated with 20 ng/ml of IL-1α and IL-1β. This suggested that 20 ng/ml of IL-1α and IL-1β were the optimal concentration (108). Then, this study varies concentration of cytokines in 2, 20, and 40 ng/ml

This study had showed that cytokine-induces apoptosis in erythroid progenitor cells of healthy subjects, β-thalassemia/hemoglobin E and anemia of chronic disease, as defined by total cell count, cell viability and cell apoptosis. The total cell count was increased in time dependent manner. However, cells treated with cytokines, IL-1β, TNF-α and IFN-γ, showed decrease cell count in all three groups.

Anemia of chronic disease showed the lowest of total cell count, which cause from pathophysiology of the disease that blunted erythropoietin to stimulate erythroid progenitor cells proliferation. In addition, the cell viability in cytokine treated cells was decreased in time dependent manner. β-thalassemia/hemoglobin E and anemia of chronic disease had lower cell count and cell viability than healthy subjects. Moreover, The percentage of erythroid progenitor cells apoptosis was increased in time dependent manner. β-thalassemia/HbE had higher apoptosis than anemia of chronic disease and healthy subjects may due to hemolysis and ineffective erythropoiesis of the disease.

There is a report about nitric oxide involved in apoptotic signaling pathway in many cell types (12, 26). Nitric oxide is an important intercellular signaling molecule involved in the regulation of diverse physiological and pathological mechanisms. It is a free oxygen radical and it act as a cytotoxic agent in pathological processes. The overproduction of nitric oxide by iNOS is important in inflammation and apoptotic process (2). The evidence showed that increased proinflammatory cytokines such as IL-1β and TNF-α can induce iNOS expression. Expression of iNOS in response to cytokine stimulation was originally observed in macrophage where it is involved in ·NO-mediated cell damage and apoptosis. Subsequent data have showed that other cell types are capable of expressing iNOS on stimulation with cytokines or microbial products. TNF-α stimulation results in NF-kB activation of corneal endothelium cell which is known to upregulate iNOS (106). IFN-γ also induces iNOS expression through activation of JAK, STAT-1 and IFN-γ response factor (IRF-1) proteins (107). However, cytokines may exert toxic effects on the cells by inducing the production of nitric oxide (·NO) and play role in induction apoptosis of erythroid progenitor cells from aplastic anemia and leukemia patients (26).

As demonstrate in this study, the effect of cytokines, IL-1β, TNF-α and IFN-γ on apoptosis in β-thalassemia/hemoglobin E and anemia of chronic disease are mediated through the induction of iNOS expression and subsequent nitric oxide generation. In β-thalassemia/hemoglobin E showed the highest effect of iNOS expression and ·NO production after treated with cytokines compared with healthy subjects and anemia of chronic disease correlated with apoptosis results. Interestingly,

cytokine showed the lowest effect on apoptosis of erythroid progenitor cells from healthy subjects correlated with low level of iNOS expression and nitric oxide production. The highest correlation was shown in IFN-γ treated cells. The positive correlation between erythroid progenitor cells apoptosis- nitric oxide production and erythroid progenitor cells apoptosis-iNOS mRNA expression were R^2=0.981 and R^2=0.988, respectively. Moreover, the negative correlation was found between hemoglobin level of sample-nitric oxide production and hemoglobin level-iNOS mRNA expression from erythroid progenitor cells treated with IFN-γ were R^2=0.757 and R^2=0.722, respectively. Then, low hemoglobin level was found in sample has high nitric oxide production. However, this correlation value lesser than 0.95, that is acceptable value may from not only nitric oxide but also other factors could be involved in severity of anemia.

Therefore, it is the possible that the apoptotic effect of cytokines is usually mediated by the induction of iNOS, which occurs in associated with high levels of ·NO production in pathological processes. There are document explain about iNOS expression and nitric oxide production. Have many factors such as cytokines and endotoxin induce iNOS expression. This study showed cytokine was statistic significantly increase iNOS mRNA expression and apoptosis in β-thalassemia/hemoglobin E and anemia of chronic disease patients compared with untreated cells (control). It may cause from defect at cytokine receptor or signaling pathways which associated with iNOS gene expression in patients. For example, cytokine, which have biphasic effect in anti-apoptosis and apoptosis though iNOS gene expression and nitric oxide production. In disease, it may have disturbances in reduce cell proliferation and exhibit cell apoptosis due to induce iNOS gene expression more than normal condition. The iNOS expression and apoptosis was also correlate in many diseases such as cardiovascular and muscleskeleton disease (41, 54).

From results of this study could suggest that cytokines, IL-1β, TNF-α, and IFN-γ involve erythroid progenitor cells apoptosis by upregulated iNOS gene expression lead to increase nitric oxide production. This mechanism might play role in pathophysiology of β-thalassemia/hemoglobin E and anemia of chronic disease.

CHAPTER VII
CONCLUSION

The present study described the cytokines, IL-1β, TNF-α, and IFN-γ induce erythroid progenitor cells apoptosis of healthy subjects, β-thalassemia/HbE and anemia of chronic disease. These cytokines decrease cell count, cell viability and increase percentage of cell apoptosis. The highest percentage of erythroid progenitor cells apoptosis was found in 20 ng/ml IFN-γ treated with erythroid progenitor cells from β-thalassemia/hemoglobin E. The results demonstrate that NO is involved in cytokine-mediated cell apoptosis determined by increase iNOS mRNA expression and nitrite concentration. Moreover, this phenomenon was confirmed by using iNOS inhibitor, SMT, which prevented both NO production and apoptosis. Interestingly, in β-thalassemia/hemoglobin E, erythroid progenitor cells treated with cytokines showed higher apoptosis and NO production than anemia of chronic disease and healthy subjects. It is possible that cytokines induced erythroid progenitor cells apoptosis mediated nitric oxide correlated with the severity of anemia in disease. It conclude that, cytokines could be induced apoptosic signaling pathway, which play role in pathogenesis of β-thalassemia/HbE and anemia of chronic disease. The knowledge from this research could be benefit for understood pathogenesis of β-thalassemia/hemoglobin E and anemia of chronic disease (Figure 45).

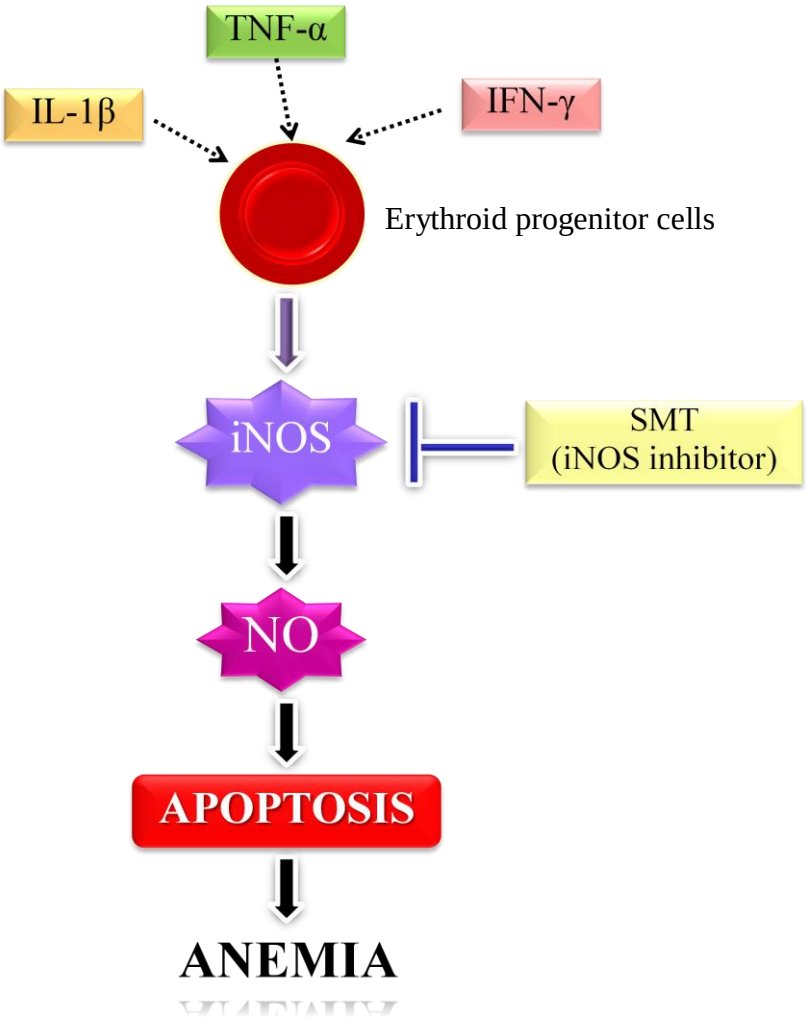


Figure 45. Possible pathways of cytokine-induced apoptosis through stimulus iNOS expression and nitric oxide production, which play role in pathogenesis of diseases.

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APPENDICES

APPENDIX A

1. Reagent for cell cultivation

1.1 A liter of Iscove's modified Dulbecco medium (incomplete medium)

Measure out 200 ml of sterile distilled water into 1000 ml beaker. Add powdered medium and gentle stirring. Rinse out inside of package to remove all traces of powder. Add 3.024 g of $NaHCO_3$ per liter of medium. Add 2% of penicillin streptomycin antibiotic solution. Add distilled water to 1 L. Stir until dissolved. Keep container closed until medium is filterd. Sterilize immediately by membrane filtration with positive pressure. Keep cold in 4˚C refrigerator.

2. Reagent for cell count

2.1 Trypan blue 0.4%

Adding 0.4 g of trypan blue into 100 ml of distill water. The solution was mixed completely and filtered before keep in the bottle at room temperature.

APPENDIX B

1. cDNA synthesis master mix

   combine the following in a 0.2 or 0.5 ml tube:

Component	Amount
10X RT buffer	2 µl
25 mM MgCl2	4 µl
0.1 M DTT	2 µl
RNaseOUT (40 U/µl)	1 µl
SuperScript III RT (200 U/µl)	1 µl

2. Real-time PCR master mix

Component	Amount
iQ SYBR Green Supermix	25 µl
Sense primer (10 µM)	2 µl
Antisense primer (10 µM)	2 µl
cDNA from control RNA	2 µl
DEPC-treated water	19 µl
Final volume	50 µl

APPENDIX C

Data of erythroid progenitor cells count from healthy subjects, β-thalassemia/ hemoglobin E and anemia of chronic disease at days 0, 3, 7, 10 and 14.

Days	Number of erythroid progenitor cells count					
	Healthy subject		β-thalassemia/HbE		Anemia of chronic disease	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
0	1±0.0		1±0.0		1±0.0	
3	0.92±0.68	0.09	1±0.0	0.12	1±0.0	0.11
7	36.78±12.29	0.00	20.98±4.07	0.00	2.84±0.53	0.03
10	199.15±30.49	0.00	106.67±16.4	0.00	9.39±1.47	0.01
14	241.5±25.9	0.00	192.33±20.22	0.00	15.79±2.94	0.00

APPENDIX D

Data of total cell count

The erythroid progenitor cells from healthy subjects, β-thalassemia/HbE and anemia of chronic disease were cultured and treated with IL-1β, TNF-α and IFN-γ in various concentration and incubation time. After incubation time, total cell count were analysed by trypan blue staining and untreated cell use as control.

Cytokines	Total cell count of erythroid progenitor cells from healthy subjects									
	Day 0		Day 3		Day 7		Day 10		Day 14	
	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*
Control	1±0.00		0.92± 0.68		36.78± 12.29		199.15± 30.49		241.5± 25.9	
IL-1β 2 ng/ml	1±0.00	1.00	0.54± 0.25	0.09	25.89± 6.44	0.00	187.22± 23.74	0.00	224.5± 22.83	0.00
IL-1β 20 ng/ml	1±0.00	1.00	1.06± 0.64	0.13	22.72± 5.6	0.00	161.06± 28.64	0.00	188.2± 27.99	0.00
IL-1β 40 ng/ml	1±0.00	1.00	1.01± 0.6	0.12	21.94± 8.26	0.00	160.02± 36.74	0.00	200.2± 20.05	0.00
TNF-α 2 ng/ml	1±0.00	1.00	0.35± 0.05	0.10	26.67± 7.8	0.00	201.17± 28.94	0.00	198.6± 23.47	0.00
TNF-α 20 ng/ml	1±0.00	1.00	0.31± 0.09	0.09	23.39± 6.08	0.00	162.94± 27.99	0.00	173.0± 18.25	0.00
TNF-α 40 ng/ml	1±0.00	1.00	0.47± 0.33	0.08	25.48± 9.7	0.00	176.59± 20.3	0.00	187.2± 10.6	0.00
IFN-γ 2 ng/ml	1±0.00	1.00	0.24± 0.06	0.07	20.87± 5.88	0.00	178.71± 33.38	0.00	173.2± 18.26	0.00
IFN-γ 20 ng/ml	1±0.00	1.00	0.17± 0.09	0.07	19.64± 4.12	0.00	143.41± 20.47	0.00	162.0± 21.72	0.00
IFN-γ 40 ng/ml	1±0.00	1.00	0.20± 0.25	0.07	20.04± 2.35	0.00	135.39± 114.47	0.00	152.6± 18.14	0.00

Cytokines	Total cell count of erythroid progenitor cells from β-thalassemia/Hemoglobin E									
	Day 0		Day 3		Day 7		Day 10		Day 14	
	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*
Control	1±0.00		1±0.00		20.98± 4.07		106.67± 16.4		192.3± 20.22	
IL-1β 2 ng/ml	1±0.00	1.00	1±0.00	1.00	18.9± 5.51	0.00	89.6± 14.74	0.00	132.4± 25.73	0.00
IL-1β 20 ng/ml	1±0.00	1.00	1±0.00	1.00	16.26± 4.59	0.00	51.06± 19.51	0.00	71.75± 24.96	0.00
IL-1β 40 ng/ml	1±0.00	1.00	1±0.00	1.00	17.58± 2.5	0.00	48.15± 18.19	0.00	75.25± 17.65	0.00
TNF-α 2 ng/ml	1±0.00	1.00	1±0.00	1.00	19.5± 2.46	0.00	83.98± 7.8	0.00	83.32± 14.08	0.00
TNF-α 20 ng/ml	1±0.00	1.00	1±0.00	1.00	16.88± 3.33	0.00	53.15± 11.09	0.00	53.25± 16.76	0.00
TNF-α 40 ng/ml	1±0.00	1.00	1±0.00	1.00	16.26± 1.41	0.00	47.45± 7.61	0.00	59.52± 14.09	0.00
IFN-γ 2 ng/ml	1±0.00	1.00	1±0.00	1.00	17.15± 1.92	0.00	75.25± 7.8	0.00	66.33± 22.4	0.00
IFN-γ 20 ng/ml	1±0.00	1.00	1±0.00	1.00	14.09± 3.15	0.00	61.09± 3.04	0.00	55.42± 10.1	0.00
IFN-γ 40 ng/ml	1±0.00	1.00	1±0.00	1.00	16.32± 1.82	0.00	55.2± 3.76	0.00	49.48± 10.89	0.00

Cytokines	Total cell count of erythroid progenitor cells from anemia of chronic disease									
	Day 0		Day 3		Day 7		Day 10		Day 14	
	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*
Control	1±0.00		1±0.00		2.84± 0.53		9.39± 1.47		15.79± 2.94	
IL-1β 2 ng/ml	1±0.00	1.00	1±0.00	1.00	2.2± 0.12	0.08	7.61± 1.86	0.02	12.47± 1.75	0.00
IL-1β 20 ng/ml	1±0.00	1.00	1±0.00	1.00	1.69± 0.23	0.04	5.41± 1.74	0.00	10.27± 1.63	0.00
IL-1β 40 ng/ml	1±0.00	1.00	1±0.00	1.00	1.67± 0.37	0.04	4.9± 0.56	0.00	11.56± 0.62	0.00
TNF-α 2 ng/ml	1±0.00	1.00	1±0.00	1.00	2.12± 0.58	0.06	6.72± 1.51	0.00	10.41± 1.24	0.00
TNF-α 20 ng/ml	1±0.00	1.00	1±0.00	1.00	1.63± 0.19	0.04	4.51± 0.39	0.00	6.34± 1.74	0.00
TNF-α 40 ng/ml	1±0.00	1.00	1±0.00	1.00	1.4± 0.14	0.03	4.12± 0.17	0.00	7.13± 1.02	0.00
IFN-γ 2 ng/ml	1±0.00	1.00	1±0.00	1.00	1.3± 0.31	0.03	4.97± 0.54	0.00	5.19± 0.94	0.00
IFN-γ 20 ng/ml	1±0.00	1.00	1±0.00	1.00	0.67± 0.04	0.01	3.83± 0.34	0.00	4.8± 0.5	0.00
IFN-γ 40 ng/ml	1±0.00	1.00	1±0.00	1.00	1.06± 0.09	0.02	3.37± 0.22	0.00	3.92± 0.26	0.00

APPENDIX E

Data of cell viability

The erythroid progenitor cells from healthy subjects, β-thalassemia/HbE and anemia of chronic disease were cultured and treated with IL-1β, TNF-α and IFN-γ in various concentration and incubation time. After incubation time, cell viability were analysed by trypan blue staining and untreated cell use as control.

Cytokines	Total cell viability of erythroid progenitor cells from healthy subjects									
	Day 0		Day 3		Day 7		Day 10		Day 14	
	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*
Control	100± 0.00		100± 0.00		100± 0.00		90.05± 1		84.62± 2.63	
IL-1β 2 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	88.47± 6.67	0.08	80.26± 5.57	0.04
IL-1β 20 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	83.27± 3.1	0.03	75.17± 2.53	0.00
IL-1β 40 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	84.2± 2.48	0.04	77.97± 3.85	0.00
TNF-α 2 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	89.43± 4.07	0.08	79.51± 3.46	0.00
TNF-α 20 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	84.74± 3.2	0.04	75.95± 4.18	0.00
TNF-α 40 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	85.86± 2.78	0.05	76.92± 7.4	0.00
IFN-γ 2 ng/ml	100± 0.00	1.00	100± 0.00	1.00	99.51± 1.09	0.16	87.5± 1.68	0.05	81.22± 8.92	0.04
IFN-γ 20 ng/ml	100± 0.00	1.00	100± 0.00	1.00	98.67± 2.98	0.11	82.76± 2.52	0.03	73.01± 6.72	0.00
IFN-γ 40 ng/ml	100± 0.00	1.00	100± 0.00	1.00	98.89± 2.49	0.10	82.19± 2.29	0.03	71.82± 9.2	0.00

Cytokines	Total cell viability of erythroid progenitor cells from β-thalassemia/hemoglobin E									
	Day 0		Day 3		Day 7		Day 10		Day 14	
	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean±SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*
Control	100± 0.00		100± 0.00		100± 0.00		85.91± 3.86		80.68± 7.13	
IL-1β 2 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	82.41± 6. 8	0.05	76.21± 5.04	0.00
IL-1β 20 ng/ml	100± 0.00	1.00	100± 0.00	1.00	99± 1.73	.016	79.29± 6.82	0.00	68.21± 4.95	0.00
IL-1β 40 ng/ml	100± 0.00	1.00	100± 0.00	1.00	98.64±2. 36	0.15	81.15± 6.83	0.02	70.32± 6.79	0.00
TNF-α 2 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	82.3± 4.33	0.03	69.82± 9.89	0.00
TNF-α 20 ng/ml	100± 0.00	1.00	100± 0.00	1.00	99.45±0. 96	0.16	79.02± 7.84	0.00	62.37± 9.11	0.00
TNF-α 40 ng/ml	100± 0.00	1.00	100± 0.00	1.00	98.02±2. 11	0.14	81.52± 3.23	0.02	65.29± 2.59	0.00
IFN-γ 2 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	81.03± 7.28	0.02	72.43± 10.76	0.00
IFN-γ 20 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	78.32± 9.14	0.00	65.51± 4.66	0.00
IFN-γ 40 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	79.68± 8.97	0.00	68.26± 10.99	0.00

Cytokines	Total cell viability of erythroid progenitor cells from anemia of chronic disease									
	Day 0		Day 3		Day 7		Day 10		Day 14	
	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*	Mean± SD	*p-value*
Control	100± 0.00		100± 0.00		100± 0.00		90.59± 4.82		83.38± 1.51	
IL-1β 2 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	88.74± 7.45	0.03	78.02± 14.14	0.00
IL-1β 20 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	82.33± 4.78	0.00	73.54± 0.2	0.00
IL-1β 40 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	81.5± 0.31	0.00	74.11± 21.21	0.00
TNF-α 2 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	87.64± 7.07	0.02	78.2± 1.68	0.00
TNF-α 20 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	85.36± 5.02	0.00	75.67± 16.52	0.00
TNF-α 40 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	83.75± 8.84	0.00	74.12± 14.14	0.00
IFN-γ 2 ng/ml	100± 0.00	1.00	100± 0.00	1.00	100± 0.00	1.00	86.43± 5.06	0.01	80.57± 12.84	0.02
IFN-γ 20 ng/ml	100±0. 00	1.00	100± 0.00	1.00	100± 0.00	1.00	83.31± 10.88	0.00	73.26± 20.31	0.00
IFN-γ 40 ng/ml	100±0. 00	1.00	100± 0.00	1.00	100± 0.00	1.00	83.67± 11.79	0.00	73.69± 18.28	0.00

APPENDIX F

Data of percentage of erythroid progenitor cell apoptosis

After treatment and harvest time, cells were stain with Annexin-V-FITC and Glycophorin A and analysed by flow cytometry. The percentage of Annexin V-FITC intensity was measured and compare with control.

Cytokines	% cell apoptosis of erythroid progenitor cell from healthy subjects					
	Day 7		Day 10		Day 14	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
Control	0±0.0		0±0.0		0±0.0	
IL-1β 2 ng/ml	1.95±0.55	0.09	1.66±0.64	0.09	3.62±0.8	0.08
IL-1β 20 ng/ml	4.03±1.05	0.07	5.37±0.79	0.07	6.25±0.76	0.06
IL-1β 40 ng/ml	2.6±0.76	0.08	4.51±0.31	0.07	5.37±0.94	0.07
TNF-α 2 ng/ml	2.81±0.96	0.08	1.59±0.45	0.09	3.96±0.76	0.07
TNF-α 20 ng/ml	5.23±0.68	0.07	6.98±1.05	0.05	8.24±1.16	0.03
TNF-α 40 ng/ml	5.0±0.49	0.07	5.29±0.65	0.07	7.24±0.48	0.04
IFN-γ 2 ng/ml	4.16±1.24	0.07	2.99±0.69	0.08	6.76±1.6	0.06
IFN-γ 20 ng/ml	6.45±1.3	0.06	5.41±1.02	0.07	8.66±0.69	0.01
IFN-γ 40 ng/ml	6.6±0.64	0.05	6.22±0.75	0.06	8.38±0.75	0.01

Cytokines	% cell apoptosis of erythroid progenitor cell from β-thalassemia/HbE					
	Day 7		Day 10		Day 14	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
Control	0±0.0		0±0.0		0±0.0	
IL-1β 2 ng/ml	0.61±0.3	0.11	3.75±0.48	0.7	2.52±0.26	0.08
IL-1β 20 ng/ml	3.14±0.11	0.08	10.48±0.2	0.01	10.71±0.53	0.01
IL-1β 40 ng/ml	2.76±0.27	0.08	8.74±0.33	0.03	8.83±0.29	0.03
TNF-α 2 ng/ml	1.43±0.28	0.09	3.54±0.87	0.07	10.7±0.52	0.01
TNF-α 20 ng/ml	3.67±0.23	0.07	12.19±1.25	0.00	16.13±1.24	0.00
TNF-α 40 ng/ml	3.62±0.16	0.07	9.68±0.79	0.02	16.04±1.07	0.00
IFN-γ 2 ng/ml	2.7±0.21	0.08	6.5±0.96	0.06	11.58±0.03	0.00
IFN-γ 20 ng/ml	5.39±0.56	0.07	12.84±1.24	0.00	17.22±1.55	0.00
IFN-γ 40 ng/ml	5.47±0.37	0.07	12.64±0.61	0.00	15.91±0.17	0.00

Cytokines	% cell apoptosis of erythroid progenitor cell from anemia of chronic disease					
	Day 7		Day 10		Day 14	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
Control	0±0.0		0±0.0		0±0.0	
IL-1β 2 ng/ml	0.7±0.01	0.11	1.65±0.5	0.09	4.06±0.4	0.07
IL-1β 20 ng/ml	1.94±0.45	0.09	6.05±0.95	0.06	8.3±0.74	0.03
IL-1β 40 ng/ml	2.48±0.27	0.08	6.85±0.25	0.05	7.66±0.54	0.05
TNF-α 2 ng/ml	0.57±0.11	0.11	3.51±0.64	0.07	7.68±0.44	0.05
TNF-α 20 ng/ml	2.78±0.06	0.08	6.02±0.98	0.06	10.11±0.53	0.00
TNF-α 40 ng/ml	2.91±0.43	0.08	6.57±0.51	0.05	10.4±0.81	0.00
IFN-γ 2 ng/ml	2.6±0.16	0.08	3.31±0.33	0.08	7.45±0.83	0.05
IFN-γ 20 ng/ml	2.71±0.11	0.08	5.51±0.8	0.06	12.15±1.87	0.00
IFN-γ 40 ng/ml	4.02±.24	0.07	5.59±0.06	0.06	12.05±0.72	0.00

APPENDIX G

Data of iNOS mRNA expression

After treatment and harvest time, RNA were extracted by Trizol reagent and determined iNOS mRNA expression by Real-time PCR.

Cytokines	iNOS mRNA expression of erythroid progenitor cell from healthy subjects					
	Day 7		Day 10		Day 14	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
Control	0±0.0		0±0.0		0±0.0	
IL-1β 20 ng/ml	1.13±0.41	0.08	1.63±0.07	0.08	2.11±0.18	0.07
TNF-α 20 ng/ml	1.62±0.17	0.08	2.6±1.11	0.07	2.16±0.74	0.07
IFN-γ 20 ng/ml	2.48±0.22	0.07	3.59±0.62	0.06	4.61±0.45	0.06

Cytokines	iNOS mRNA expression of erythroid progenitor cell from β-thalassemia/HbE					
	Day 7		Day 10		Day 14	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
Control	0±0.0		0±0.0		0±0.0	
IL-1β 20 ng/ml	6.65±1.26	0.04	8.52±1.27	0.02	14.54±3.72	0.00
TNF-α 20 ng/ml	1.01±0.48	0.09	6.12±1.39	0.04	10.46±1.04	0.00
IFN-γ 20 ng/ml	7.66±0.47	0.03	9.95±2.26	0.01	20.72±1.04	0.00

Cytokines	iNOS mRNA expression of erythroid progenitor cell from anemia of chronic disease					
	Day 7		Day 10		Day 14	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
Control	0±0.0		0±0.0		0±0.0	
IL-1β 20 ng/ml	0.6±0.14	0.10	3.08±0.17	0.06	4.45±1.1	0.04
TNF-α 20 ng/ml	0.81±0.18	0.10	2.73±1.54	0.07	3.26±0.22	0.06
IFN-γ 20 ng/ml	0.72±0.04	0.10	3.39±0.3	0.06	6.15±1.2	0.04

APPENDIX H

Data of percentage of nitric oxide production

After treatment and harvest time, cells were centrifuged to get supernanant and the level of nitrite concentration were quantified by griess assay.

Cytokines	Nitrite concentration of erythroid progenitor cell from healthy subjects					
	Day 7		Day 10		Day 14	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
Control	0±0.0		0±0.0		0±0.0	
IL-1β 20 ng/ml	980.56± 19.97	0.00	901.2± 168.23	0.00	781.71± 60.03	0.00
TNF-α 20 ng/ml	823.35± 9.97	0.00	830.03± 101.26	0.00	741.2± 78.89	0.00
IFN-γ 20 ng/ml	1009.54± 98.6	0.00	892.79± 169.03	0.00	860.17± 20.1	0.00

cytokines	Nitrite concentration of erythroid progenitor cell from β-thalassemia/HbE					
	Day 7		Day 10		Day 14	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
Control	0±0.0		0±0.0		0±0.0	
IL-1β 20 ng/ml	950.13± 9.94	0.00	1403.6± 169.89	0.00	1372.63± 130.27	0.00
TNF-α 20 ng/ml	751.02± 119.68	0.00	1538.75± 61.31	0.00	1624.11± 9.96	0.00
IFN-γ 20 ng/ml	702.73± 108.83	0.00	1748.16± 150.2	0.00	1513.49± 141.15	0.00

Cytokines	Nitrite concentration of erythroid progenitor cell from anemia of chronic disease					
	Day 7		Day 10		Day 14	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
Control	0±0.0		0±0.0		0±0.0	
IL-1β 20 ng/ml	902.57± 129.79	0.00	795.51± 70.04	0.00	1064.1± 59.38	0.00
TNF-α 20 ng/ml	776.83± 69.6	0.00	898.67± 30.07	0.00	1091.22± 59.98	0.00
IFN-γ 20 ng/ml	918.72± 121.02	0.00	919.75± 19.97	0.00	1132.23± 40.01	0.00

APPENDIX I

Data of percentage of cell apoptosis treated with iNOS inhibitor

After erythroid progenitor cells treated with cytokines and selective inhibitor of iNOS (SMT), cells were stain with Annexin V-FITC and Glycophorin A and analysed by flow cytometry. The percentage of Annexin V-FITC intensity was measured and compared with control.

Cytokines	% cell apoptosis of erythroid progenitor cell from healthy subjects at Day 7		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	4.03±1.05	1.92±0.87	0.11
TNF-α 20 ng/ml	5.23±0.68	2.33±1.6	0.13
IFN-γ 20 ng/ml	6.45±1.3	2.27±0.41	0.03

Cytokines	% cell apoptosis of erythroid progenitor cell from healthy subjects at day 10		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	5.37±0.79	3.74±0.99	0.10
TNF-α 20 ng/ml	6.98±1.05	3.04±0.23	0.04
IFN-γ 20 ng/ml	5.41±1.02	1.84±0.78	0.03

Cytokines	% cell apoptosis of erythroid progenitor cell from healthy subjects at day 14		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	6.25±0.76	1.26±1.24	0.01
TNF-α 20 ng/ml	8.24±1.16	2.34±0.56	0.01
IFN-γ 20 ng/ml	8.66±0.69	1.55±1.12	0.00

Cytokines	% cell apoptosis of erythroid progenitor cell from β-thalassemia/HbE at day 7		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	3.14±0.11	0.97±0.67	0.06
TNF-α 20 ng/ml	3.67±0.23	1.5±0.3	0.12
IFN-γ 20 ng/ml	5.39±0.56	1.53±0.61	0.02

Cytokines	% cell apoptosis of erythroid progenitor cell from β-thalassemia/HbE at day 10		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	10.48±0.6	3.49±1.43	0.02
TNF-α 20 ng/ml	12.19±1.25	2.48±1.6	0.01
IFN-γ 20 ng/ml	12.84±1.24	4.27±1.86	0.00

Cytokines	% cell apoptosis of erythroid progenitor cell from β-thalassemia/HbE at day 14		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	10.71±1.53	3.84±1.14	0.00
TNF-α 20 ng/ml	16.13±1.24	5.26±1.45	0.01
IFN-γ 20 ng/ml	17.22±2.55	5.56±1.67	0.00

Cytokines	% cell apoptosis of erythroid progenitor cell from anemia of chronic disease at day 7		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	1.94±0.45	0.32±0.08	0.19
TNF-α 20 ng/ml	2.78±0.06	0.96±0.23	0.22
IFN-γ 20 ng/ml	2.71±0.11	0.79±0.44	0.16

Cytokines	% cell apoptosis of erythroid progenitor cell from anemia of chronic disease at day 10		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	6.05±0.95	3.12±0.39	0.06
TNF-α 20 ng/ml	6.02±0.98	3.39±1.01	0.06
IFN-γ 20 ng/ml	5.51±0.8	2.18±0.57	0.02

Cytokines	% cell apoptosis of erythroid progenitor cell from anemia of chronic disease at day 14		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	8.3±0.74	3.88±0.5	0.00
TNF-α 20 ng/ml	10.11±0.53	3.54±1.09	0.00
IFN-γ 20 ng/ml	12.15±1.87	3.64±1.13	0.00

APPENDIX J

Data of iNOS mRNA expression (with iNOS inhibitor)

After erythroid progenitor cells treated with cytokines and selective inhibitor of iNOS (SMT), RNA were extracted by trizol reagent and determined iNOS mRNA by Real-time PCR.

Cytokines	iNOS mRNA expression of erythroid progenitor cell from healthy subjects at day 7		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	1.13±0.41	0.57±0.31	0.08
TNF-α 20 ng/ml	1.62±0.17	0.43±0.17	0.06
IFN-γ 20 ng/ml	2.48±0.22	0.91±0.61	0.04

Cytokines	iNOS mRNA expression of erythroid progenitor cell from healthy subjects at day 10		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	1.63±0.07	0.61±0.33	0.13
TNF-α 20 ng/ml	2.6±1.11	0.89±0.58	0.04
IFN-γ 20 ng/ml	3.59±0.62	1.11±0.27	0.04

Cytokines	iNOS mRNA expression of erythroid progenitor cell from healthy subjects at day 14		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	2.11±0.18	0.88±0.12	0.09
TNF-α 20 ng/ml	2.16±0.74	1.01±0.3	0.06
IFN-γ 20 ng/ml	4.61±0.45	1.2±0.51	0.03

Cytokines	iNOS mRNA expression of erythroid progenitor cell from β-thalassemia/HbE at day 7		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	6.65±1.26	4.1±0.47	0.09
TNF-α 20 ng/ml	1.01±0.48	0.45±0.15	0.10
IFN-γ 20 ng/ml	7.66±0.47	1.08±0.29	0.04

Cytokines	iNOS mRNA expression of erythroid progenitor cell from β-thalassemia/HbE at day 10		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	8.52±0.27	1.59±0.28	0.01
TNF-α 20 ng/ml	6.12±0.39	1.62±0.34	0.02
IFN-γ 20 ng/ml	9.95±0.26	1.31±0.65	0.01

Cytokines	iNOS mRNA expression of erythroid progenitor cell from β-thalassemia/HbE at day 14		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	14.54±1.72	4.5±1.1	0.02
TNF-α 20 ng/ml	10.46±1.04	5.34±0.75	0.02
IFN-γ 20 ng/ml	20.72±1.04	5.5±0.58	0.01

Cytokines	iNOS mRNA expression of erythroid progenitor cell from anemia of chronic disease at day 7		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	0.6±0.14	0.05±0.2	0.13
TNF-α 20 ng/ml	0.81±0.18	0.0±0.07	0.10
IFN-γ 20 ng/ml	0.72±0.04	0.0±0.16	0.11

Cytokines	iNOS mRNA expression of erythroid progenitor cell from anemia of chronic disease at day 10		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	3.08±0.17	0.84±0.07	0.04
TNF-α 20 ng/ml	2.73±1.54	1.33±0.48	0.08
IFN-γ 20 ng/ml	3.39±0.3	0.92±0.13	0.03

Cytokines	iNOS mRNA expression of erythroid progenitor cell from anemia of chronic disease at day 14		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	4.45±1.1	1.78±0.14	0.03
TNF-α 20 ng/ml	3.26±0.22	1.5±0.31	0.04
IFN-γ 20 ng/ml	6.15±1.2	2.34±0.26	0.03

APPENDIX K

Data of percentage of nitric oxide production (with iNOS inhibitor)

After erythroid progenitor cells treated with cytokine and selective inhibitor of iNOS (SMT), cells were centrifuged to get supernatant and the levels of nitrite production were quantified by griess assay.

Cytokines	Nitrite concentration of erythroid progenitor cell from healthy subjects at day 7		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	980±20	620±69	0.04
TNF-α 20 ng/ml	823±10	520±81	0.05
IFN-γ 20 ng/ml	1009±98	660±74	0.03

Cytokines	Nitrite concentration of erythroid progenitor cell from healthy subjects at day 10		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	901±168	430±48	0.00
TNF-α 20 ng/ml	830±101	420±17	0.01
IFN-γ 20 ng/ml	892±169	450±28	0.01

Cytokines	Nitrite concentration of erythroid progenitor cell from healthy subjects at day 14		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	781±60	260±22	0.04
TNF-α 20 ng/ml	741±78	170±10	0.04
IFN-γ 20 ng/ml	860±20	280±15	0.02

Cytokines	Nitrite concentration of erythroid progenitor cell from β-thalassemia/HbE at day 7		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	950±10	320±63	0.02
TNF-α 20 ng/ml	751±119	290±71	0.02
IFN-γ 20 ng/ml	702±108	260±39	0.06

Cytokines	Nitrite concentration of erythroid progenitor cell from β-thalassemia/HbE at day 10		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	1403±169	460±93	0.00
TNF-α 20 ng/ml	1538±61	400±32	0.00
IFN-γ 20 ng/ml	1748±150	530±51	0.00

Cytokines	Nitrite concentration of erythroid progenitor cell from β-thalassemia/HbE at day 14		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	1372±130	370±47	0.00
TNF-α 20 ng/ml	1624±10	510±45	0.00
IFN-γ 20 ng/ml	1513±141	160±35	0.00

Cytokines	Nitrite concentration of erythroid progenitor cell from anemia of chronic disease at day 7		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	902±130	230±18	0.03
TNF-α 20 ng/ml	776±70	290±16	0.04
IFN-γ 20 ng/ml	918±121	200±27	0.03

Cytokines	Nitrite concentration of erythroid progenitor cell from anemia of chronic disease at day 10		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	795±70	70±21	0.02
TNF-α 20 ng/ml	898±30	110±27	0.02
IFN-γ 20 ng/ml	919±20	180±51	0.02

Cytokines	Nitrite concentration of erythroid progenitor cell from anemia of chronic disease at day 14		
	Without iNOS inhibitor	With iNOS inhibitor	*p-value*
	Mean ± SD	Mean ± SD	
Control	0.0±0.0	0.0±0.0	
IL-1β 20 ng/ml	1064±60	210±15	0.02
TNF-α 20 ng/ml	1091±60	340±63	0.03
IFN-γ 20 ng/ml	1132±40	110±32	0.00

APPENDIX L

Documentary proof of ethical clearance

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MAHIDOL UNIVERSITY
Since 1888
Siriraj Institutional Review Board

Certificate of Approval

COA no.Si 550/2009

Protocol Title : Effects of cytokines on nitric oxide mediated erythroid precursor cells apoptosis of β-thalassemia/Hemoglobin E and anemia of chronic disease

Protocol number : 468/2552(EC4)

Principal Investigator/Affiliation : Assist.Prof.Dalina Tanyong, M.D.
Faculty of Medical Technology, Mahidol University

Research site : Faculty of Medicine Siriraj Hospital

Approval includes :

1. SIRB Submission Form
2. Proposal
3. Participant Information Sheet
4. Informed Consent Form
5. Case Record Form

Approval date : November 4, 2009

Expired date : November 3, 2010

This is to certify that Siriraj Institutional Review Board is in full Compliance with International Guidelines For Human Research Protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

(Prof. Jariya Lertakyamanee, M.D.)
Chairperson

November 6, 2009
date

(Clin. Prof. Teerawat Kulthanan, M.D.)
Dean of Faculty of Medicine Siriraj Hospital

November 12,2009
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BIOGRAPHY

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