# EFFECT OF MITHRAMYCIN&HYDROXYUREA ON FETAL HEMOGLOBIN PRODUCTION IN CULTURED ERYTHROID CELLS FROM PATIENTS WITH β-THALASSEMIA.

## Thesis

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By
**AHMED MOHAMED ALI**
M.B.B.Ch Cairo University

Supervised By

**Prof.Dr. SHAHIRA AMIN ZAYED**
Professor of Clinical and Chemical Pathology
Cairo University

**Dr. KHALED MOHAMED SALAMA**
Ass.Professor of Pediatrics
Cairo University

**Dr. RANIA FAWZY HAMMOUD**
Ass.Professor of Clinical and Chemical Pathology
Cairo University

Faculty of Medicine
Cairo University
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# *Abstract*

We report in this study that the DNA-binding drug Mithramycin is a potent inducer of fetal hemoglobin (HbF) production in erythroid cells from healthy human subjects and β-thalassemia patients.

Erythroid precursors derived from peripheral blood were grown in 2-phase liquid culture. In this procedure, early erythroid progenitors proliferate and differentiate during phase 1 (in the absence of erythropoietin) into late progenitors. In phase 2, in the presence of erythropoietin, the latter cells continue their proliferation and mature into Hb-containing orthochromatic normoblasts. Compounds were added on days 4 to 5 of phase 2 (when cells started to synthesize Hb), and cells were harvested on day 12.



Key Words :

Haematopoiesis - Augmentation of Fetal Hemoglobin Production

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# CONTENTS

Page

# *Introduction*

It is well established that increase of fetal hemoglobin (HbF) to 30% of the total hemoglobin leads to a significant improvement of the clinical status of patients affected by β thalassemia, and so the biosynthesis of HbF inplace of deficient HbA could be a suitable treatment for β haemoglobinopathies. **( *Rodgers GP, Rachmilewitz EA. 1996* )**

Several pharmacological agents such as Azacytidine , Hydroxyurea , Erythropoietin and Butyrate Analogs enhance fetal hemoglobin production and have been used in hemoglobinopathy patients to ameliorate severe pain episodes and reduce severe anemia. ***( Olivieri NF, Weatherall DJ. 1998 )***

In this respect, DNA-binding drugs, which are known to modify the formation of DNA nuclear protein complexes and thereby control gene expression, appear to be of great interest in augmentation of HbF production. ***( Olivieri NF, Weatherall DJ 1998 )***

Many researches have recently demonstrated that Hydroxyurea and Mithramycin are powerful inducers of erythroid differentiation of the human leukemic K562 cell line, and are potent inducers of gamma globin mRNA accumulation and fetal Hb production in erythroid cells from healthy human

subjects and β thalassemic patients. ***( Weatherall DJ, Stamatoyannopoulos G, 2001 )***

The pattern of erythroid differentiation and γ globin gene expression could be influenced by treatment with DNA-binding drugs. Interestingly, while chromomycin binding to DNA generates stable complexes, Mithramycin -DNA complexes are highly unstable. This could explain the low toxicity of Mithramycin as compared with chromomycin. For this reason, Mithramycin was proposed as a therapeutic agent in several neoplastic diseases such as chronic myelogenous leukemia and testicular cancer ***( Weatherall DJ, Stamatoyannopoulos G.2001 )***

# *Aim of the work*

The main issue of this study is to test whether Mithramycin is able to augment HbF production in erythroid precursor cells from healthy human subjects as well as from β- thalassemia patients in comparison to Hydroxyurea . This is a mandatory preliminary step in the evaluation of Mithramycin as a potential drug for the development of treatment for these diseases.

# *Chapter 1 Haemoglobin*

## *NORMAL HAEMATOPOIESIS*

The physiologic process of formation of blood cells is known as haematopoiesis. It proceeds through different stages starting from early embryonic life-mesoblastic stage (yolk sac), hepatic stage, and myeloid (bone marrow) stage. During embryonic and early foetal life, haematopoiesis occurs in the yolk sac (only erythoblasts) and the liver (all blood cells). Some blood cells formation also occurs in the spleen (all blood cells), lymph nodes and thymus (mostly lymphocytes). Bone marrow starts producing blood cells around 3rd to 4th month and by birth becomes the exclusive site of blood cell formation (Fig. 1.1 ).In younger age, whole of the skeletal marrow participates in blood cell production. By late childhood, haematopoiesis becomes restricted to the flat bones such as sternum, ribs, iliac bones and vertebrae and proximal ends of long bones. At other skeletal sites haematopoietic areas are replaced by fat cells. However, when there is increased demand for blood cell production, conversion of yellow fatty inactive marrow to red active marrow can occur. In extremely severe cases (e.g. severe chronic anemia), resumption of haematopoietic activity in organs other than bone marrow such as liver and spleen (extramedullary haematopoiesis) can occur.***( Spangrude GJ. Et al, 1994)***

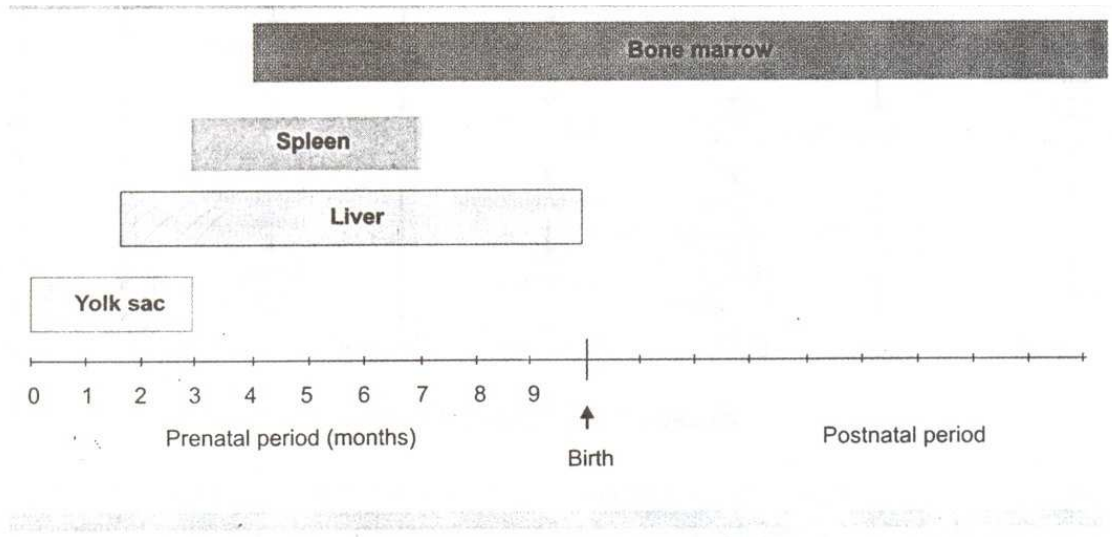


**FIGURE 1.1:** Stages of haematopoiesis

## RED BLOOD CELLS

### *Stages of Erythropoiesis*

The earliest morphologically identifiable erythroid cell in the bone marrow is the proerythroblast (pro-normoblast), a large (15-20µm) cell with a fine, uniform chromatin pattern, one or more nucleoli, and dark blue cytoplasm.The next cell in the maturation process is the basophilic (early) normoblast. This cell is smaller in size (12-16 µm) and has a coarser nuclear chromatin with barely visible nucleoli. The cytoplasm is deeply basophilic. ***( Iligh KA, et al, 2002 )***

The more differentiated erythroid cell is the polychromatic (intermediate) normoblast (size 12- 15µm). The nuclear size is

smaller and the chromatin becomes clumped. Polychromasia of cytoplasm results from admixture of blue ribonucleic acid and pink haemoglobin. This is the last erythroid precursor capable of mitotic division. ***( Iligh KA, et al, 2002 )***

The orthochromatic (late) normoblast is 8 to 12 µm in size. The nucleus is small, dense and pyknotic and commonly eccentrically-located. The cytoplasm stains mostly pink due to haemoglobinization. It is called as orthochromatic because cytoplasmic staining is largely similar to that of erythrocytes. The nucleus is ultimately expelled from the orthochromatic normoblast with the formation of a reticulocyte. The reticulocyte still has remnants of ribosomal RNA in the form of a cytoplasmic reticulum. After ١ to 2 days in the bone marrow and 1-2 days in peripheral blood reticulocytes lose RNA and become mature pink-staining erythrocytes ***( Iligh KA, et al, 2002 )***

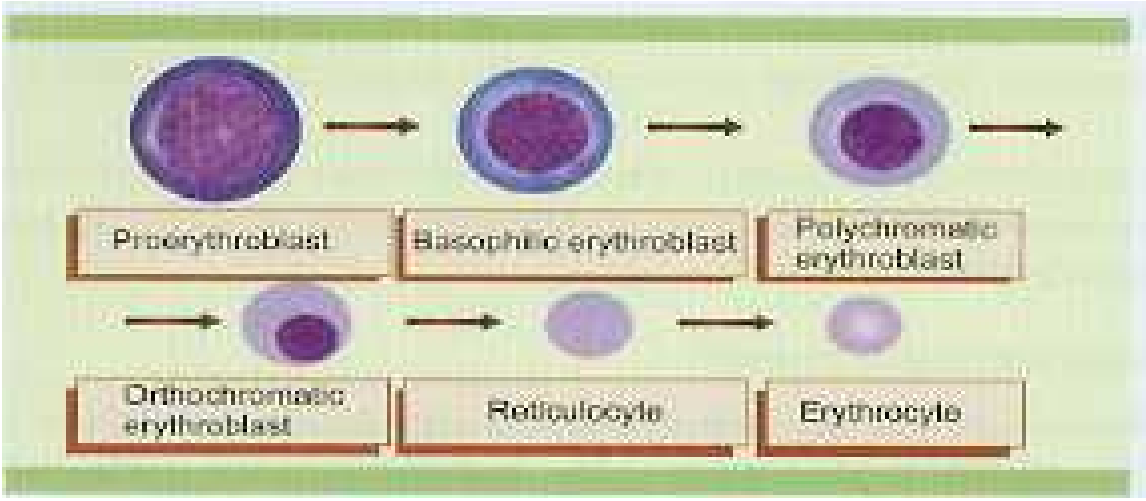


Figure 1.2: Stages in the formation of a mature red cell, with each stage cell size and nuclear size become smaller, cromatin clumping increases and ultimately nucleus is extruded, color of cytoplasm gradually changes from basophilic to orange red.

About four mitotic divisions and continued differentiation lead to the production of 16 mature erythrocytes from each pronormoblast. ***( Iligh KA, et al, 2002 )***

### *Structure and Function of Erythrocytes*

Mature erythrocyte is a round biconcave disc about 7 to 8µm in diameter. Basic structural properties of various red cell components (haemoglobin, enzymes, and membrane) are outlined below:

### *Haemoglobin*

Haemoglobin is responsible for transport of oxygen from lungs to the tissues and of carbon dioxide from tissues to the lungs.

# *Chapter 1 Haemoglobin*

Haemoglobin (MW 64,500 daltons) is composed of haem (consisting of iron and protoporphyrin) and globin. The globin portion of the molecule consists of four (or two pairs of) polypeptide chains. One haem group is bound to each polypeptide chain.***( Mohandas N, et al. 1993 )***

***Normal variants of haemoglobin****:*

Haemoglobin is not homogeneous and normally different variants exist such as A, A2, F, Gower I, Gower II, and Portland. The last three are present only during embryonic life. Others are present in varying proportions during foetal and adult life. The relative proportions of different haemoglobins are: Adults-HbA 97 % , Hb A2 2.5 %, and HbF O.5 %,; Newborns-HbF 80% and HbA 20%.***( Kaufman RE. et al. 2001.)***

Haemoglobin A (HbA), the principle haemoglobin of adults, consists of a pair each of alpha (α) and of beta (β) polypeptide chains and its structure is designated as α2β2.

Foetal haemoglobin (HbF), the predominant haemoglobin in foetal life, contains a pair of alpha (α) and a pair of gamma (γ) chains. Two types of chains are distinguished, G γ A γ, which have different amino acids (either glycine or alanine) at position 136. Thus, HbF is heterogeneous and contains α2γ2 136Gly and α2γ2

136 Ala. ***( Kaufman RE. et al. 2001.)***

During embryonic life, there are three haemoglobins: Gower I ( ζ2ε2 ) Gower II ( α2ε2 ) and Portland ( ζ2γ2 ). With foetal development, synthesis of zeta ( ζ ) and epsilon ( ε ) chains is replaced by that α and γ chains respectively. Afterbirth, production of γ chains switches to that of β and delta ( δ ) chains***.( Weatherall DJ ,et al. 2001 )***

***Strucure of globin genes :***

Normal haemoglobin is a tetramer composed of a pair of α-like and a pair of β-like polypeptide chains. Each chain is linked to one molecule of haem. The α-like polypeptide chains (ζ and α) and β-like polypeptide chains (ε, γ, β, and δ) are encoded by α and β-globin gene clusters on chromosomes 16 and 11 respectively. The order of genes in α-globin gene cluster from 5' 10 3' end is ζ-ψζ-ψα2-ψα1-α2-α1. The order of genes in β-globin gene cluster from 5' to 3' end is ε-Gγ-Aγ-ψβ-δ-β (Fig. 1.3). The ψζ,ψζ2,ψα1,and ψβ are pseudogenes. A pseudogene (ψ) contains sequences similar to a functional gene but is rendered inactive due to mutation during evolutionary process. ***( Iligh KA, et al, 2002 )***

In humans, autosomal chromosomes occur in pairs. As each member of chromosome 16 has two α gene loci ("locus refers to

specific physical position of a gene on chromosome), there are total four α genes. However, there is only one β globin gene locus on chromosome 11, and therefore β genes are two in number. ***( Iligh KA, et al, 2002 )***

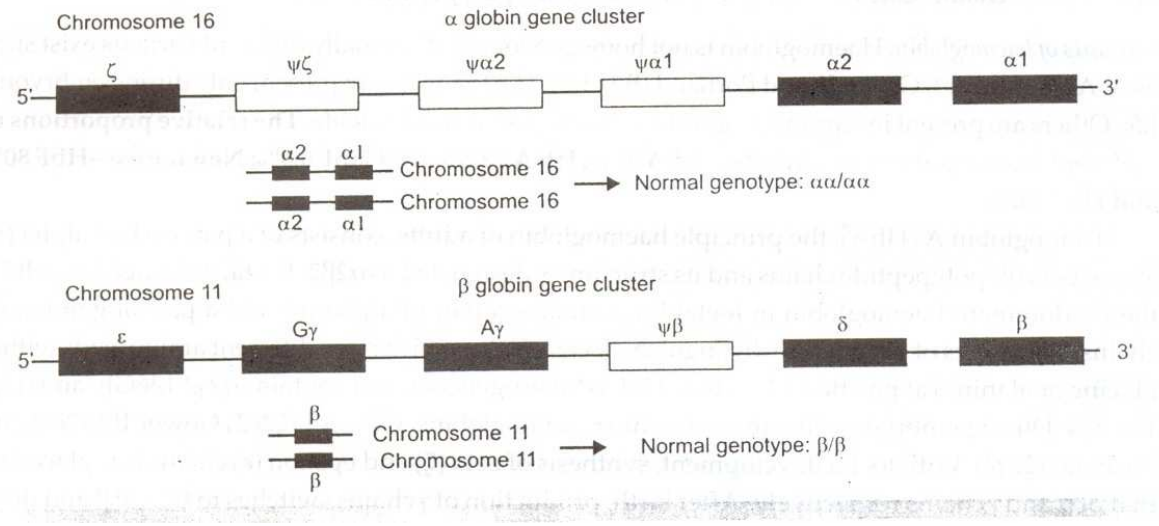


Figure 1.٣ : α and β genes clusters .normal genotype is shown below each gene cluster

Genes are the base sequences, which are present along the DNA strands and are necessary for the formation of a protein. The different functional areas of a globin gene are:

(1) Exons and introns : The regions of DNA strand which encode amino acids in the protein product are known as exons while non-coding regions which interrupt the coding sequences are known as introns or intervening sequences. Each globin gene contains three exons and two introns. ***(Shirish M Kawthalkar , 2006)***

(2) Splice junction sequences: These are sequences at the junction of exons and introns and are required for precise splicing (or removal) of introns during the formation of mRNA. ***(Shirish M Kawthalkar , 2006)***

(3) Promoter: The promoter region is present towards 5' end of the gene and contains sequences to which the RNA polymerase binds; it is necessary for correct initiation of transcription. Two promoter sequences are TATA and CCAAT. ***(Shirish M Kawthalkar , 2006)***

(4) Polyadenylation signal: The 3' end of the globin gene contains the sequence AATAAA that serves as a signal for the addition of a poly-A track to the mRNA transcript (Fig. 1.٤). ***(Shirish M Kawthalkar , 2006)***

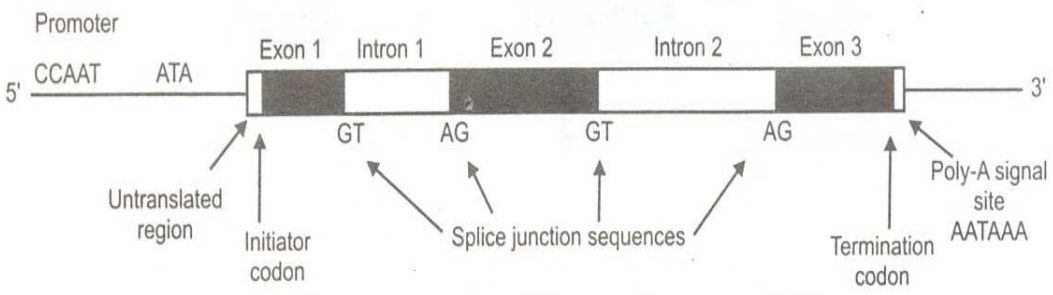


Figure 1.٤: A schematic diagram of β globin gene