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Ineffective Erythropoiesis: Associated Factors and Their Potential as Therapeutic Targets in Beta-Thalassaemia Major

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Authors' contributions

This work was carried out in collaboration between all authors. Authors HA and RH contributed to the conception, design and writing of this paper. Authors BAZ and SS contributed to critically revising the manuscript regarding important intellectual content. All authors read and approved the final manuscript.

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ABSTRACT

Beta-thalassaemia (β-thal.) is single-gene disorder that exhibits much clinical variability. β-thal. major is a major health problem, and the only method of curing is allogenic bone-marrow transplantation, which is not available to everyone and not without risk.

The underlying pathogenesis of β-thal. major is due to ineffective erythropoiesis (IE), which is characterized by increased proliferative activity that fails to produce sufficient functional red blood cells. In β-thal. patients, the severity of the IE is mainly responsible for the hallmarks of the disease's presentation, sequalae and complications.

This review discusses the mechanisms of IE, the factors that contribute to it and the potential therapies for targeting these factors to improve patients' clinical phenotypes.

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ABBREVIATIONS

DSbs : DNA double –strand breaks
EMH : Extramedullary haematopoiesis
EPO : Erythropoietin
EpoR : Erythropoietin receptor
FPP : Fermented papaya preparation
GVHD : Graft versus host disease
Hb : Haemoglobin
HDR : Homology- directed repair
HSCs : Haematopoietic stem cells
HSCT : Haematopoietic stem cells transplantation
HSP70 : Heat shock protein 70
IE : Ineffective erythropoiesis
Jak2 : Janus kinase-2
LPO : Lipid peroxidation
NHEJ : Non homologous end- joining
ROS : Reactive oxygen species
SCFAs : Short-chain fatty acids
SCID-X1 : X-linked severe immunodifeciency disorder
STAT5 : Signal transducer and activator of transcription-5
Thal : Thalassaemia
TALENs : Transcription activator–like effector nucleases
ZFNs : Zinc finger nucleases

1. INTRODUCTION TO β-THALASSAEMIA

Beta-Thalassaemia (β-thal.) is one of the commonest single-gene disorders worldwide [1]. In Southeast Asia, most β -thal. cases are autosomal recessive, although autosomal dominant cases are seen rarely. Each human carries two β-globin genes on chromosome 11. One defective gene results in asymptomatic carriers, and if both genes are defective, the patient will be born alive (unlike in α-thal.), but will need lifelong transfusion support [2].

Haemoglobin (Hb) in the red blood cells is responsible for transporting oxygen from the lungs to the tissues and for transporting carbon dioxide from the tissues back to the lungs. Hb comprises two main parts: heme, which contains iron and porphyrin; and protein part represent the globins. Globin in red blood cells comprises four subunits: two identical α-globins and two identical non-α globins (γ,δ,ε or β) [3]. HbA, which consists of two α- and two β-chains, represents 98% of the total Hb in adults, while HbA2, which consists of two α- and two γ-chains, represents the remaining 2% of the Hb in adults [4]. In thalassaemia, there is a reduction in or absence of one or more of these globin chains [5].

β-thal. is classified into thalassaemia major (also called Cooley anaemia), intermedia and minor, according to the disease's severity. Thalassaemia major is fatal if untreated, and currently, the only therapy that cures it is an allogenic bone-marrow transplant (HSCT). However, allogenic HSCT is not without risks and is not available to most patients, as finding a compatible donor is not easy. Genetically modified, autologous haematopoietic stem cells (HSCs) may be a better solution than allogenic HSCT, as HSCs precludes the need to find a suitable donor. In addition, HSCs presents no immunological risks, as the transplanted cells used are the patient's own [6].

The main characteristics of β-thal. are severe anaemia, ineffective erythropoiesis (IE) and extramedullary haematopoiesis (EMH). Patients require lifelong blood transfusions to sustain life [7]. In a milder form of the disease, β-thal. intermedia, iron overload is also seen, but regular blood transfusions are not required. In the intermedia form, increased iron absorption from the gastrointestinal tract can reach three to four times the normal rate, ranging from two to five grams per year, depending on the degree of erythroid expansion. IE usually increases over time, aggravating the anaemia, iron absorption and splenomegaly. Increased gastrointestinal iron absorption also contributes to iron overload among β-thal. major patients who are regularly transfused. Consequently, iron is deposited in many organs, leading to organ damage and death if not properly treated [8].

2. BIOLOGY OF INEFFECTIVE ERYTHRO-POIESIS

IE is an abnormal type of erythropoiesis that is characterized by insufficient production of mature erythrocytes despite a massive increase in erythroid proliferation in the bone marrow [9]. When β-globin genes are defective, the immature erythroid cells fail to reach full maturity and undergo haemolysis prematurely. To compensate for this loss, the erythroid precursors expand. This results in further production of defective cells and worsening of the condition, causing marrow expansion and

EMH, which presents as splenomegaly and skeletal deformities like frontal bossing [8,10,11]. This early cell death or apoptosis is a feature of IE [12].

Among many factors that contribute to IE in β-thal, oxidant injury generated by excess α-globin chains plays an important role in apoptosis. In normal erythropoiesis, before binding to other globin chains, α-globin complex with a chaperone called haemoglobin stabilizing protein (AHSP) that prevents misfolding of α-globin. Studies have shown that AHSP and β-globin compete to bind to α-globin at the same binding region, called the $\alpha_1\beta_1$ interface, but the binding affinity of β-globin is greater and can displace AHSP to form HbA [13]. In β-thal., production of α-globin chains is normal. However, due to the deficit of β-globin chains, the α-globin accumulates in the erythroid precursors. These accumulated α-globin chains exceed the AHSP capacity and form tetramers, which precipitate in the red cells. These tetramers form inclusion bodies that undergo auto-oxidation, forming hemichromes and generating reactive oxygen species (ROS), which cause further damage by releasing toxic cellular contents that cause further damage and extensive intramedullary haemolysis of cells before they reach full maturation (Fig. 1.) [8,9,11,14-16].

Accumulation of α-globin and ROS can change cells' deformability and hydration, resulting in cytoskeletal damage. ROS affects both protein 4.1 and transmembrane band 3 protein. Band 3 clustering under oxidant stress produces an antigen that binds IgG and both complements and attracts phagocytic macrophages [4].

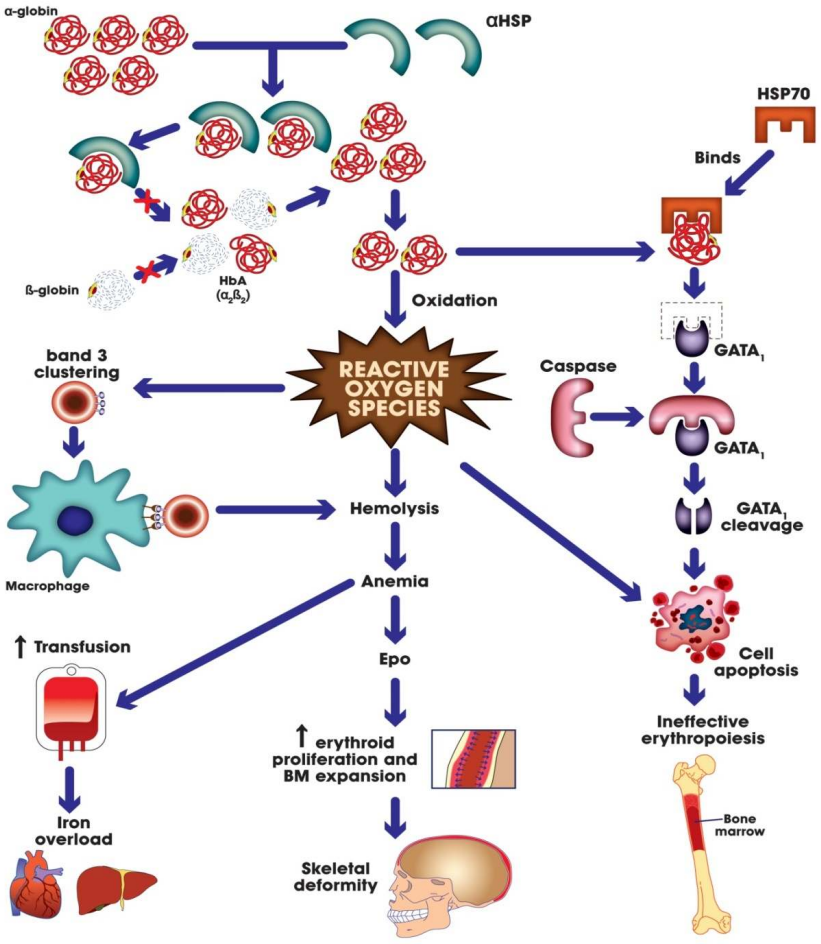

Fig. 1. Pathophysiology and complications of β- thalassemia

Table 1. Factors that may contribute to IE

AHSP sequestration
α-globin accumulation in RBCs
ROS formation
Oxidation of protein 4.1 and band 3
GATA1 binding to caspase-3

Fas and FasL are death receptors expressed at early stage of differentiation. Both are down-regulated in thalassaemic mice compared to normal mice. This can be a result of erythropoietic stress and may play a role in erythroid expansion [17].

Heat shock protein 70 (HSP70) is a molecular chaperone that is regulated by erythropoietin (EPO). EPO facilitates the entry of HSP70 into the erythroblast nucleus, where it binds to GATA1, a main transcription factor in erythropoiesis, and protects it from transiently activated caspase-3. In β-thal., accumulated free α-globin binds to HSP70, exposing GATA1 without protection, which causes end-stage maturation arrest and apoptosis [18]. In the bone marrow, this maturation arrest and apoptosis result in IE and anaemia, which stimulate EPO production, which in turn further induces erythroid proliferation and bone marrow expansion [19]. In patients who need regular transfusions, iron overload cannot be avoided.

3. POTENTIAL THERAPIES FOR FACTORS CONTRIBUTING TO INEFFECTIVE ERYTHROPOIESIS

IE results from both apoptosis and limited differentiation of the bone marrow's highly proliferating erythroid precursors. Studies have shown that many factors can interact to cause various levels of IE. One option for potential therapy is targeting those factors separately or collectively to control the disease's severity. The following discusses therapy options for targeting IE.

3.1 Controlling the Primary Defect

Restoring sufficient production of β-globin can protect patients from the sequelae of the disease's progression, which are discussed below.

3.1.1 Haematopoietic stem cell transplantation

Allogenic HSCT is the only known curative treatment for β-thal. However, there are many limitations and challenges to this option. To be eligible for allogenic HSCT, the patient should be in the early stage of the disease, have few comorbidities, and have a compatible donor willing to go through the donation process. Even when these conditions are met, the process is not without risk. Because transplantation from a leucocyte antigen (HLA)-identical family donor stills carry a 5% mortality. In addition, the high risk of graft rejection or failure and the high risk of growth and gonadal failure all necessitate the availability of other options [20].

3.1.2 Gene therapy

Adding globin genes to a patient's own cells by using a viral vector has shown great progress in recent years. Using the patient's own cells helps to eliminate the risk of graft versus host disease (GVHD) and the need for a compatible donor for allogenic HSCT. However, to be the treatment of choice, gene therapy must meet the criteria of any successful therapy by being safe and specific and providing a persistent, sufficient level of β–globin production [20]. Unfortunately, safety issues are still a significant challenge to this mode of therapy. Concern has increased since four out of nine children with X-linked severe immunodeficiency disorder (SCID-X1) developed leukaemia after gene therapy using a retrovirus vector [21].

3.1.3 Genome editing

Genome editing, previously known as gene targeting, uses targetable endonucleases to induce DNA double-strand breaks (DSbs) in specific loci in the targeted gene, stimulating endogenous repair mechanisms. Genome editing can be used to stimulate gene targeting or stimulate mutations by small insertions or deletions. Endogenous repair takes place in the cell by means of one of two repair pathways: non-homologous end-joining (NHEJ) or homology-directed repair (HDR) [22]. There are four main types of engineered nucleases: mega-nucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR-associated nucleases (Cas9). Mega-nucleases, ZFNs and TALENs bind to the targeted DNA through protein-DNA interaction, while the Cas9 protein can target new DNA sequences using a small RNA molecule that can base-pair with DNA [23,24].

Many studies have shown that genome editing to be a potential therapeutic option in thalassaemia

and sickle-cell anaemia [25-27]. Unlike in gene therapy, in genome editing, there is no risk of insertional mutagenesis. However, genome editing is known to induce genomic instability, which may cause translocations and chromosomal aneuploidy. Further investigation and assessment of these methods are needed [22].

3.2 Controlling Apoptosis

3.2.1 Targeting alpha chain excess

In normal red blood cells, α-chains bind to β-chains to form dimers of α/β, and tetramers of α_2/β_2 are formed when two dimers combine, giving rise to HbA, which is the main Hb type in adults. In β-thal., an imbalance between α– and non-α, which results from the absence of sufficient β-globin, results in the α-chains being precipitated in the red blood cells, damaging the cells' membranes and leading to early cell death. This mechanism plays a significant role in the pathophysiology of IE in β-thal.

Any factor that reduces the α/non-α imbalance is known to reduce disease severity. Of great interest are the following.

1. Reducing α-chain production. Co-inheritance of α-thal. with β-thal. alleviates the α/non-α imbalance by reducing α-chain production. The gene down-regulation approach has been adapted by many studies of β-thal. to reduce α-chain production. For example, in-vivo and in-vitro RNA interference using viral or nonviral vectors or a pharmacological approach using epigenetic drugs have been used, and these methods have shown that α-chain reduction is associated with improvement of the phenotype [28,29]. However, some questions must be clarified, including the safety of the various types, the effect of gene therapy on nontargeted genes and the ability of these methods to achieve the desired level of down-regulating α-chains, as reducing α-chains below the desired level can worsen the anaemia [30,31].
2. Increasing the HbF level. In this case, the production of α-chains is not affected but the excess α-chains bind to γ-chains, forming HbF. This can be achieved in many ways, including the following.
   a. Pharmacological agents: Studies have used medications to induce endogenous foetal globin (γ-globin) to increase HbF in both β-thal. and sickle-cell anaemia [32, 33]. Chemotherapy, including hydroxyurea and 5-azacytidine, have increased HbF but are known for to be cytotoxic. Short-chain fatty acids (SCFAs) are considered safer than chemotherapy but are rapidly metabolized and must be used in large doses [20,33-35]. However, using these therapy options offers no permanent cure, as maintenance is required.
   b. Gene therapy: At birth, HbF represents the main Hb type, but the level declines throughout the first year of life until HbF is greatly reduced and HbA becomes the main Hb type. The HbF level can be increased by inducing the gamma (γ) gene itself or targeting its promoters or repressors [36].

3.2.2 Reducing oxidative stress

Oxidative stress is an important factor in β-thal. pathophysiology. Many factors cause ROS and lipid peroxidation (LPO) to be hyper-produced until they exceed the ability of antioxidants to overcome the damage process in the cells and the oxidation of the red blood cells in β-thal. major, as outlined in Fig. 2 [37].

In β-thal., release of ROS results mainly from two pathological mechanisms: excessive accumulation of the highly oxidant, unpaired α-globin chains and release of highly toxic free iron from damaged cells, which stimulates the redox reaction, depleting reduction ability. Together, these mechanisms result in membrane instability and greater oxidization of the Hb, with clusters of red cell membrane proteins, including band 3, increasing red blood cell damage. Oxidative stress has also been found to play an indirect role in removal of red blood cells by macrophages, which recognize the clustered band 3 under the effects of oxidization. Furthermore, oxidative stress can activate K-Cl transport, causing decreased intercellular K^+ and consequent water loss [31,38].

Antioxidants are known to be substances that can significantly delay or suppress oxidation but not eliminate it entirely. Therefore, they reverse the damage caused by free radicals before they can react with the surrounding tissues. Antioxidants can be divided into natural and synthetic [39].

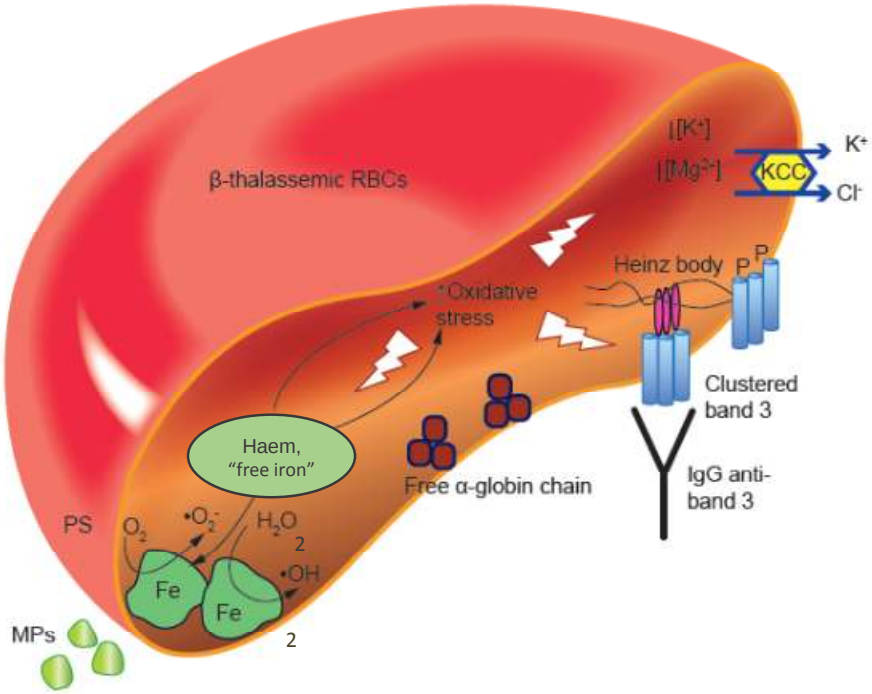

Fig. 2. Schematic diagram of abnormalities observed in *β*-thalassemic red cells

It is known that oxidative insult can affect the life span of red blood cells, but its effect on IE has not been investigated extensively. Fermented papaya preparation (FPP) has been shown to decrease oxidative stress in red blood cells, lipid peroxidation and the phagocytic index of red blood cells, which decreases cell removal by the phagocytic system [38]. Another type of antioxidant, resveratrol, has been found to induce erythroid maturation, reduce IE and oxidative stress and increase haemoglobin levels, which makes it a potential therapy option [40].

3.2.3 Protection of HSP70 protein

Erythropoiesis is a complex process regulated by the interplay of many transcriptional factors, including GATA1, which regulates erythroid development, maturation and survival by regulating erythroid-specific genes, including the erythropoietin receptor (EpoR) gene. As part of the normal maturation and differentiation process, transient activation of caspase occurs, inducing condensation of nuclear chromatin and loss of cellular organelles, which results in expulsion of the nucleus from the cell without causing cell death [4,41]. Without the protection of HSP70, GATA1 is degraded by caspase-3, leading to maturation arrest and cell death [42].

A study by Arlet et al. [18] found that HSP70 was sequestrated by free α-globin chains and was therefore no longer available to protect GATA1, resulting in apoptosis. Using a lentivirus that expressed mutant HSP70, a wild-type HSP70 was introduced into β-thal. major CD34+ cells, improving the terminal maturation of the erythroblasts and decreasing the apoptosis two-fold compared with cells transduced by empty lentivirus. This could be a potential therapy option targeting IE.

4. CONTROLLING BONE-MARROW EXPANSION AND SUBOPTIMUM DIFFERENTIATION

As mentioned earlier, IE causes chronic anaemia, bone-marrow expansion, growth retardation, extramedullary erythropoiesis, iron overload and organ damage. Bone-marrow expansion and suboptimum differentiation are discussed below.

4.1 Targeting Janus Kinase-2 (Jak2)

Jak2 is a member of the Jak tyrosine kinase family, and the *Jak2 gene* is found on chromosome 9. Lack of *Jak2* in mice causes embryonic death due to defective erythropoiesis [43]. Jak2 phosphorylation occurs when EPO

binds to EpoR, activating complex signalling pathways including signal transducers, including transcription-5 (STAT5), which in turn activates many genes responsible for erythroid-cell proliferation and differentiation.

The study of various diseases in humans has shown that increased Jak2 activation can lead to enormous increases in erythropoiesis in a pattern similar to that observed in β-thal. These diseases include polycythaemia vera, essential thrombocythaemia and chronic idiopathic myelofibrosis. All these diseases have been shown to include increased Jak2 activity due to (V617F) mutation [44].

Experiments using Jak2 inhibitors on thalassemic mice have reduced not only IE but also spleen size, which indicates that Jak2 inhibitors may be a potential therapeutic option for β- thal., especially for those patients with milder forms of the disease, as this option is safer for them than receiving blood transfusions throughout childhood to maintain normal growth. The study tested only the uncontrolled erythropoiesis element of IE and did not consider erythroid apoptosis, which also contributes to IE, or whether using Jak2 inhibitors affects the rate of apoptosis [12].

A recent study found that a hormone called erythroferrone suppressed hepcidin and, subsequently, iron absorption. Hepcidin suppression resulted in iron overload, which, in turn, initiated oxidative damage and apoptosis. One potential therapy option could be targeting erythroferrone to eliminate its suppressive effect on hepcidin. Interestingly, it has been proposed that this hormone is regulated by EPO through the Jak2-STAT5 pathway [45-47].

5. CONCLUSIONS

Recent research has revealed much about β-thalassaemia that has increased understanding of the disease's pathophysiology. This advance, in parallel with advances in therapy technologies, holds the promise of a cure for thalassaemia.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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