Usefulness of Eosin-5'-Maleimide (EMA) Dye for the Diagnosis of Hereditary Spherocytosis

Thesis

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Contents

Subjects	Page

- **List of Abbreviations** ..... **I**
- **List of Tables** ..... **III**
- **List of Figures** ..... **IV**
- **Introduction** **1**
- **Aim of the Work** **4**
- **Review of Literature**
  - Chapter (I): Hemolytic Anemias **5**
  - Chapter (II): Methods of Diagnosis of Hereditary Spherocytosis **38**
- **Subjects and Methods** **60**
- **Results** **68**
- **Discussion** **87**
- **Summary** **96**
- **Conclusion** **101**
- **Recommendations.** **102**
- **References.** **104**
- **Appendices.** **126**
- **Arabic Summary** ..... **---**

List of Abbreviations

Abb.	Full term
AD	Autosomal dominant
AGLT	Acidified glycerol lysis test
AIHA	Autoimmune hemolytic anemia
Ala	Alanine
ANK1	Ankyrin 1-gene
AR	Autosomal recessive
Asp	Aspartic acid
BM	Bone marrow
BTI	Beta thalassemia intermedia
CBC	Complete blood count
CD	Cluster of differentiation
CDAII	Congenital dyserythropoietic anemia type II
CT	Computed tomography
DAT	Direct antiglobulin test
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
EMA	Eosin maleimide test
EMH	Extra medullary hematopoiesis
FITC	Fluorescein isothiocyanate
G6PD	Glucose-6-phosphate dehydrogenase
HA	Hemolytic anemia
Hb	Hemoglobin
HBf	Hemoglobin F
Hct	Hematocrit
HE	Hereditary elliptocytosis

Abb.	Full term
HPP	Hereditary pyropoikilocytosis
HPV	Human papilloma virus
HS	Hereditary spherocytosis
HSV	Herpes simplex virus
HUS	Hemolytic uremic syndrome
IDA	Iron deficiency anemia
LDH	Lactate dehydrogenase
MCF	Mean cell fluorescence
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean cell volume
MFI	Median fluorescence intensity
MM	Multiple myeloma
OFT	Osmotic fragility test
OHSt	Over hydrated hereditary stomatocytosis
PBS	Phosphate buffer saline
PVP	Polyvinylpyrrolidone
RBC	Red blood cell
RDW	Red cell distribution width
RES	Reticuloendothelial system
Rh	Rhesus
SA/V	Surface area to volume
SAO	South-east Asian ovalocytosis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TTP	Thrombotic thrombocytopenic purpura
WBC	White blood cell

List of Tables

No	Table	Page
1	Probable primary causes for membrane protein defects	19
2	Characteristic features of Hereditary Spherocytosis according to age group	26
3	Clinical classification of severity of Hereditary Spherocytosis	27
4	Asymptomatic persons with conspicuous laboratory parameters	28
5	Indications for splenectomy	34
6	Demographic and clinical data of different types of anemia in this study	77
7	Laboratory data of control group and different types of anemia in this study	78
8	Comparison between HS patients and different types of anemia regarding clinical findings	79
9	Comparison between HS patients and each type of anemia regarding laboratory findings	79
10	Comparison between control groups and different groups of anemia patients regarding EMA MFI	80
11	Comparison between IDA, AIHA and BTI regarding EMA expression	80
12	Correlation between MFI, % reduction in EMA and laboratory parameters in HS group of patients	81
13	Multi-regression analysis that sort parameters according to their sensitivity to discriminate HS	82
14	Diagnostic validity test showing different values for EMA MFI and % fluorescence reduction and corresponding sensitivity and specificity in HS patients	83
15	Diagnostic validity test for % reduction in EMA fluorescence cutoffs for diagnosis of HS	84

List of Figures

No	Figure	Page
1	Classification of hemolytic anemia	8
2	Red cell membrane structure	12
3	Protein composition of the red blood cell membrane skeleton	15
4	Pathogenesis of spherocytosis	17
5	Membrane defects in HS affect the “vertical” interactions connecting the membrane skeleton and the lipid bilayer	18
6	Red cell in hereditary elliptocytosis and other elliptic red cell membrane disorders	36
7	Peripheral blood film of spherocytosis	40
8	Different morphological findings in spherocytosis	41
9	Morphology of HS blood film with band 3 and protein 4.2 defects	42
10	Osmotic fragility test in hereditary spherocytosis	46
11	Osmoscan curve relevant parameters: Omin, Omax, and Ohyper	53
12	Erythrocyte membrane protein profile in normal red cell membrane and hereditary spherocytosis by Fairbanks 3.5-17% exponential gradient	55
13	Chemical structure of eosin-5-maleimide	57
14	Comparison between HS cases, control group and other hemolytic/ non-hemolytic anemia patients regarding MCV and MCHC	80
15	Comparison between HS cases, control group and other hemolytic/ non-hemolytic anemia patients regarding MFI and % reduction in fluorescence	81
16	ROC curve analysis showing the diagnostic performance of EMA.MFI and %decrease in EMA fluorescence for discriminating patients with HS from those other types of anemia	85

No	Figure	Page
17	Flow cytometric analysis of EMA binding test in HS case	85
18	Flow cytometric analysis of EMA binding test in a control case	86
19	Flow cytometric analysis of EMA binding test in an AIHA case	86

Usefulness of Eosin-5'-Maleimide (EMA) Dye for the Diagnosis of Hereditary Spherocytosis

ABSTRACT

Background: the normal biconcave discoid shape of the red blood cell (RBC) is maintained by cytoskeletal proteins underneath the membrane. This protein network and its link with the lipid bilayer are essential for RBCs to remain flexible and carry out their physiological functions. A number of disorders including hereditary spherocytosis (HS) and hereditary elliptocytosis (HE) are caused by decreased expression or mutation of cytoskeletal proteins.

Aim of the Work: to evaluate the usefulness eosin-5-maleamide (EMA) dye binding flow cytometric test in the diagnosis of HS and assess the sensitivity and specificity of this test to distinguish HS patients from patients having anemia due to other causes.

Subjects and Methods: this study was conducted on 52 patients with chronic anemia, 21 diagnosed as HS (group I) and 31 patients with anemia due to other causes (group II). The patients were 28 males and 23 females with a male to female ratio 1.2:1, their ages ranged between 3 to 12.5 years old attending at the Pediatric Hematology Clinics, Ain Shams University Hospitals. Normal control group (group III) was included in the study.

Results: median fluorescence intensity (MFI) had shown significantly lower values in HS patients (group I) (3.78±0.48) than (iron deficiency anemia) IDA patients (group IIa) (5.56±0.35), auto immune hemolytic anemia (AIHA) patients (group IIb) (5.7±0.27), beta thalassemia intermedia (BTI) patients (group IIc) (6.01±0.63) and control group (group III) (5.3±0.43). The % reduction in EMA fluorescence proved to be significantly higher in HS patient (group I) (29.88±8.27) than in IDA patients (group IIa) (1.13±6.18), AIHA patients (-6.14±10.44) and BTI patients (group IIc) (-5.1±10.07). Multi regression analysis had been used to check the most sensitive predictors for diagnosis of HS, both EMA MFI and the % reduction in EMA fluorescence proved to be the best sensitive predictive values for discriminating HS patients, however the higher F-ratio in EMA MFI (F=25.222) than in % reduction in EMA fluorescence intensity (F=19.866) showed that the MFI is even better than % reduction in fluorescence in diagnosis of HS.

Conclusion: the present study highlighted the role of flow cytometric EMA binding test in differentiating HS patients from patients having anemia due to other causes.

Keywords: *Eosin-5'-Maleimide - Hereditary Spherocytosis - Autosomal Dominant*

Introduction

The normal biconcave discoid shape of the red blood cell (RBC) is maintained by cytoskeletal proteins underneath the membrane. This protein network and it's link with the lipid bilayer are essential for RBCs to remain flexible and carry out their physiological functions. A number of disorders including hereditary spherocytosis (HS) and hereditary elliptocytosis (HE) are caused by decreased expression or mutation of cytoskeletal proteins ***(Golafshan et al., 2014).***

Hereditary spherocytosis is the most common congenital hemolytic anemia (HA) in Caucasians, affecting approximately 1 in 1000-2000 individuals ***(Bianchi et al., 2012).*** The genes responsible for HS encode one or more proteins of the cytoskeleton of the RBC membrane, which are ankyrin, spectrin, pallidin (protein 4.2), and band 3 protein (anion exchanger 1) ***(Gallagher, 2004).*** Inheritance of HS is autosomal dominant (AD) in approximately two-thirds of the patients (typical HS), in which ankyrin mutations are the most common cause of HS ***(Golafshan et al., 2013).***

Hereditary spherocytosis shows marked heterogeneity, ranging from an asymptomatic condition to

fulminant HA. Presentation may be at any age. Patients with severe cases may present as neonates, while those with mild HS may not come to medical attention until adulthood, when an environmental stressor uncovers their disorder. The major complications of HS are aplastic or megaloblastic crisis, hemolytic crisis, cholecystitis and cholelithiasis ***(Bolton-maggs et al., 2012; Bianchi et al., 2012).***

Diagnosis of HS is based on clinical features, family history, peripheral smear examination and laboratory investigations. The first line screening tests for the diagnosis of HS like osmotic fragility test (OFT) based on decreased surface area to volume ratio of the spherocytic red cells determine the extent of red cell lysis over a period of time in various incubation media. However, a normal OFT does not exclude a diagnosis of HS and may occur in 10–20% of cases of HS. The test may be normal in presence of iron deficiency, obstructive jaundice and in the recovery phase of aplastic crisis affected by elevated reticulocyte count ***(Bolton-Maggs et al., 2004).***

Therefore, this test has a low sensitivity and specificity as it fails to detect atypical and mild cases and is affected by factors unrelated to red cell cytoskeletal defects ***(Kar et al., 2010).***

Other HS diagnostic methods include visual examination of RBC membrane proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) which also has some disadvantages, such as a strict requirement for expensive equipment, variable detection sensitivity depending on the nature of the defective protein, and ethnic variation ***(Won, 2009).***

A flow cytometric test, based on the fluorescence of RBCs after incubation with eosin-5'-maleimide (EMA) dye, was described for the diagnosis of HS ***(King et al., 2000).*** Studies showed that this fluorescence was mainly attributable to the band 3 protein (80%) and, to a lesser degree, to proteins associated with the Rh blood group protein complex ***(Bolton-Maggs et al., 2004)***. Further studies confirmed these previous results and proposed this test as a screening method for HS ***(Girodon et al., 2008).***

Aim of the Work

The aim of this present study was to evaluate the usefulness EMA dye binding flow cytometric test in the diagnosis of HS and assess the sensitivity and specificity of this test to distinguish HS patients from patients having anemia due to other causes to be applied in routine diagnostic workup.

Chapter (I):

Hemolytic Anemias

During its 120-day life span in performing its function of oxygen delivery to cells in various tissues, the 8-μm discoid human red cell undergoes extensive cellular deformation during its repeated passages through 2 to 3μm-diameter capillaries of the microvasculature. Decreased cellular deformability reduces the ability of the red cell to efficiently deliver oxygen to the tissues. Importantly, loss of cellular deformability leads to sequestration of the undeformable red cells primarily by the spleen leading to decreased cell life span and resultant anemia ***(Safeukui et al., 2012; Danielczok et al., 2017).***

Definition of anemia

Anemia is defined as a reduction in red blood cell (RBC) mass or blood hemoglobin concentration. Practically, anemia is commonly defined by reductions in hemoglobin (Hb), hematocrit (Hct) or both ***(Gordon-Smith and Mohandas, 2011).***

The threshold for defining anemia is Hb or Hct that is more than two standard deviations below the mean for the reference level. Normal ranges for Hb and Hct varies with age; therefore, it is particularly important to use age

and sex adjusted norms when evaluating a pediatric patient for anemia ***(Robins and Blum, 2007; Gordon-Smith and Mohandas, 2011).***

Hemolytic anemia

Anemia is considered to be hemolytic when RBCs are prematurely cleared from the circulation. Hemolytic anemia (HA) can be further subdivided into intra- or extravascular hemolytic anemia, and the underlying cause can be either inherited or acquired ***(Huisjes et al., 2018).***

Intravascular hemolysis as the name suggests, is defined as lysis of RBC inside vasculature. The cause can be hereditary, as seen in sickle cell disease or can be initiated by certain drugs, mechanical stress (for example through shear forces generated by artificial heart valves), by cold-agglutination or as a result of exhaustive exercise. Intravascular hemolysis causes the release of HB into the plasma. Free HB is toxic and can lead to various clinical manifestations, such as hemoglobinuria, renal dysfunction, pulmonary hypertension and platelet activation ***(Rother et al., 2005; Körmöczi et al., 2006; Cappellini and Fiorelli, 2008; Kato et al., 2017)***.

Extravascular hemolysis is directly related to reduced erythrocyte deformability, these RBCs fail to pass through