

In vitro study the affinity of sickle cell hemoglobin to change to methaemoglobin .

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Abstract:

This study involve (80) female medical student, aged between(19-24) years, subdivided into three groups, control group that consist of (30) female with normal hemoglobin erythrocyte genotype(Hb AA) and two case groups, each one consist of (25) female, one with(Hb AS) and the other with(HbSS) erythrocyte genotype. In this study we tried to identify the affinity of sickle erythrocyte hemoglobin to change to methaemoglobin (MetHb) in three human erythrocyte genotypes, namely,(HbAS) and (HbSS) compared with normal genotype (HbAA) . The cyanomethaemoglobin reaction was used for the determination of erythrocyte haemolysate methaemoglobin concentration. The mean \pm SD methaemoglobin concentration, expressed as percentage (MetHb%) of total Hb erythrocyte genotypes (HbAS,HbSS and HbAA). Thus, the oxidative damage of Hb diminishes among the studied genotypes in the manner: SS>AS>AA. We demonstrate a statistically significant differences ($P<0.05$) in MetHb% between (HbSS) and (HbAA) erythrocyte genotypes, whereas, there is no significant differences ($P>0.05$) in MetHb% between HbAS and HbAA erythrocyte genotypes. This results may provide further insight into the mechanism of how (MethHb)formation benefit sickle cell patients and may offer an attractive new therapeutic model for the treatment of sickle cell disease.

Keyword :hemoglobin, erythrocyte genotypes, methaemoglobin.

Introduction:

Hemoglobin (Hb) is the iron containing oxygen transport metalloprotein in the red blood cells of all vertebrates.¹ Each hemoglobin molecule composed of heme, the portion of molecule containing iron and globin, the portion made up of amino acid chains.² The iron may be either in the (Fe^{+2}) or in the (Fe^{+3}) state,³ but ferrihemoglobin (methaemoglobin) when (Fe^{+3}) which is the oxidized form of hemoglobin cannot bind oxygen.⁴ In such cases, the enzyme nicotinamide-adenine dinucleotide (NADH) methaemoglobin reductase in red blood cells will be able to eventually reactivate methaemoglobin (MethHb) by reducing the iron, so the iron must be exist in the (Fe^{+2}) oxidation state to bind oxygen.⁵ On the other hand, hemoglobin variants occur when mutations occur in the globin protein.⁶ These variants are inherited in an autosomal recessive manner, affecting people either as homozygous such as hemoglobin (SS) i.e. sickle cell disease or heterozygous such as hemoglobin (AS) i.e. sickle cell trait.⁷ In sickle cell disease, deoxygenation of sickle hemoglobin (HbS) leads to intracellular polymer formation, ultimately distorting the erythrocyte and retarding red cell transit through the micro vasculature.⁸ Therefore, sickle cell disease characterized by extensive hemolysis, increased cellular adhesion and vaso occlusion.⁹ In this study we tried to identify the affinity of sickle hemoglobin to change to methaemoglobin (MethHb) of three human erythrocyte genotypes, namely, (HbAS), (HbSS) and (HbAA), that may provide further insight into the mechanism of how (MethHb) formation benefit sickle cell patients and may offer an attractive new therapeutic model for the treatment of sickle cell disease.

Materials and Methods:

Selection of patients/ preparation of blood sample:

Venous blood sample (5ml) were obtained from (80) female medical students have been chosen randomly, aged between (19-24) years from medical college during the academic educational year 2010-2011. Investigations have been done to identify the type of hemoglobin by (hemoglobin electrophoresis method) and estimation

of hemoglobin concentration by(Sahli hemoglobinometer). They are subdivided according to the results of their investigations into three groups: control group consist of (30) female with HbAA and two case groups, one consist of (25)female with HbAS and the other consist of (25) female with HBSS. Each sample of blood were obtained from participants by venipuncture and stored in EDTA anti coagulant tubes.

Measurement of methaemoglobin(MethHb) in blood.

Principle:

Hemoglobin(Hb) has maximum absorption at (630nm). When cyanide is added, this absorption band disappears and the resulting change in absorbance is directly proportional to the concentration of Hb. Total Hb in the sample is then measured after complete conversion to HbCN by addition of ferri cyanide- cyanide reagent. The conversion will measure HbO₂ and Hb but not HbS. Thus, the presence of a large amount of HbS will result in an erroneously low measurement of total Hb.¹⁰

Method:

Lyse (0.2)ml of blood in a solution containing(4ml) of phosphate buffer (0.1mol/L, PH 6.8) and (6ml) of Non-ionic detergent solution. Divide the lysate into two equal volumes (A and B). Measure the absorbance of A in spectrophotometer at (630nm) (D1). Add one drop of potassium cyanide (50g/L) solution and measure the absorbance again, after mixing(D2). Add one drop of potassium ferricyanide (50g/L) solution to B, and after 5min, measure the absorbance at the same wave length(D3). Then add one drop of potassium cyanide solution to B and after mixing make a final reading(D4). All the measurements are made against a blank containing buffer and detergent in the same proportion as present in sample.

Calculation:

Methaemoglobin (MethHb%) = $\frac{D1-D2}{D3-D4} \times 100$

The test should be carried out within one hour of collecting the blood. After dilution, the buffered lysate can be stored for up to 24h at 2-4C° without significant auto-oxidation of Hb to(methHb).¹⁰

Statistical analysis:

The experiments were designed in a completely randomized method. The results analysis were performed with SPSS statistical software version 10. ANOVA analysis of variance, probability value of ($P < 0.05$) was considered to be statistically significant.

Results:

Table 1: visualize the mean \pm SD of hemoglobin concentration, expressed as percentage (Hbg%) of total Hb erythrocyte genotypes. There was a significant decrease ($P < 0.05$) in the concentration of Hb of (HbSS) erythrocytes in comparison with (HbAA) erythrocytes which is expectable.

This study analyses the products resulting from the oxidization of (Hb), identified by the (MethHb) concentration in three human erythrocyte genotype, namely, (HbSS, HbAS) compared with normal genotype (HbAA). Analysis of the products resulting from hemoglobin oxidation damage, characterized by an increase in the mean levels of (MethHb) which are directly related to the increase in the (HbS) concentration. The mean \pm SD methaemoglobin concentration, expressed as percentage (MethHb%) of total Hb erythrocyte genotypes, thus oxidative damage of Hb diminishes among the studied genotype in a manner: $SS > AS > AA$. There was a significant differences ($P < 0.05$) in (MethHb%) between (HbAA) and (HbSS) erythrocytes, whereas there was no significant differences ($P > 0.05$) in (MethHb%) between (HbAA) and (HbAS) erythrocytes, so the level of (MethHb) in sickle cell genotype exhibit a significant higher ($p < 0.05$) concentration comparable with (HbAA) (table2).

Table 1: Hemoglobin concentration(Hb g %) in various groups

Genotypes		No. of samples	(Hbg%) mean \pm SD
Control	HbAA	30	11.21 \pm 1.43
Group 1	HbAS	25	10.45 \pm 0.96
Group 2	HbSS	25	9.72 \pm 0.810*

*Significant decrease($p < 0.05$) compare with control group.

Table 2: Methaemoglobin concentration (MethHb%) of erythrocyte haemolysate

Genotypes	No. of samples	(MethHb%) mean±SD
Control HbAA	30	10.28±0.95
Group 1 HbAS	25	14.07±1.04
Group 2 HbSS	25	16.10±2.67*

***significant difference (P<0.05) compare with control group.**

Discussion:

It was demonstrated that (MethHB) reduction in intact red blood cells (RBCs) is depended on NADH- methaemoglobin reductase enzyme content,¹¹ and that the impaired (MethHb) reduction rate in sickle RBCs may results from a lack of increase in NADH-methaemoglobin reductase enzyme content.¹² Therefore, there is no relationship between the erythrocytes hemoglobin concentrations and their affinity to change to methaemoglobin¹³.

Previous reports have associated the dysfunctional erythrocytes (HbSS) genotype with raised level of oxidized Hb.¹⁴ They noted that the primary reason for the relatively raised concentration of oxidized Hb (MethHb) in HbSS erythrocytes was the higher production of super oxide ion by these erythrocytes compared to those of HbAS and HbAA.¹⁵ Specifically, sickle erythrocytes spontaneously generate approximately twice as much super oxide, peroxide and hydroxyl radical when compared with normal erythrocytes.¹⁶ Moreover, the potential redox couple formed by sickle cell membrane associated ferric ion and cytoplasmic oxyhemoglobin is promotive of Hb oxidation and deposition of hemichrome on the membrane.¹⁷

In addition, HbSS erythrocytes contain an increase amount of malon dialdehyde, a by-product of lipid peroxidation and evidence of abnormal amino group cross-linking by malon dialdehyde has been demonstrated in lipid extract of HbSS erythrocytes membranes.¹⁸ This phenomenon confirms the susceptibility of sickle erythrocytes to endogenous free radical- mediated oxidative damage that correlates with the proportion of irreversible sickled erythrocytes.¹⁹

The current studies shed some light on the mechanisms of inhibition of sickling by (MethHb).²⁰ It would appear that (MethHb) exerts it's chief effects by removing pigment from the equilibrium between reduced Hb and oxyhemoglobin rather than by interfering with the sickling process in a manner analogous to the interference produced by fetal Hb.²¹ While other studies suggested that (MethHb) inhibits red cell sickling and high levels of (MethHb) in the blood levels rise as high as 50% of total Hb prevent the symptoms of sickle cell disease.²²

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دراسة مختبرية للتعرف على قابلية الهيموغلوبين لكريات الدم المنجلي للتغير للنمط المؤكسد (الميتيموغلوبين)

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الخلاصة:

هذه الدراسة شملت (80) طالبة في كلية الطب. تتراوح أعمارهم بين (19-24) سنة، قسموا إلى ثلاث مجموعات، المجموعة الاولى (control) تتكون من (30) طالبة طبيعية الهيموغلوبين الوراثي لكريات الدم الحمراء (الهيموغلوبين AA)، والمجموعة الثانية (case) تتألف من مجموعتين، كل واحدة تتألف من (25) طالبة، واحدة مع (خضاب الدم AS) والأخرى مع كرات الدم الحمراء الوراثي (HbSS). في هذه الدراسة حاولنا التعرف على قابلية الهيموغلوبين لكريات الدم المنجلي للتغير إلى الميتيموغلوبين (MetHb) في ثلاثة أنماط جينية لكريات الدم الحمراء وهي: (مهاينات الناقل المضيف HbAS) و (HbSS) مقارنة مع النمط الوراثي العادي (HbAA). وقد استخدم cyanomethaemoglobin لتحديد تركيز كرات الدم الحمراء الميتيموغلوبين. تركيز الميتيموغلوبين معبرا عنه بنسبة مئوية (%MetHb) من إجمالي المورثات كرات الدم الحمراء الهيموغلوبين (مهاينات الناقل المضيف HbAS، و (HbSS) و (HbAA). وهكذا، فإن الضرر التأكسدي يقل بين الطرز الوراثية المدروسة وكما يلي: $AS < SS$ و $AA < AS$. ونحن نبرهن على وجود فروق ذات دلالة إحصائية ($P < 0.05$) في %MetHb بين (HbSS) والأنماط الجينية لكريات الدم الحمراء (HbAA)، في حين لا يوجد فروق ذات دلالة إحصائية ($P < 0.05$) في (%MetHb) بين مهاينات الناقل المضيف والأنماط الجينية كرات الدم الحمراء HbAA. قد يؤدي هذا إلى توفير مزيد من التبصر في كيفية الاستفادة من آلية تشكيل (MethHb) لمرضى فقر الدم المنجلي، وربما تقديم نماذج علاجية جديدة لعلاج مرض فقر الدم المنجلي.