In vitro study of the fragility and deformability of the sickle cell: the effects of NSAIDs and primaquine

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ABSTRACT

Background: **The sickle red blood cell differs physiologically from the normal red cell, especially in fragility and viscocity. The effects whether useful or deleterious of various drugs such as antibiotics or analgesics on the sickle red blood cell are not well investigated, especially the drugs with oxidative potential.**

Objectives: **The present study was carried out to examine the difference in osmotic fragility and deformability between normal and sickle red blood cells before and after, in vitro incubation with oxidative drugs.**

Materials and Methods: **Blood samples were obtained from sickle cell disease patients (n=39) and normal volunteers (n=19). Sickle hemoglobin was detected by electrophoresis; osmotic fragility was measured by using hypotonic sodium chloride solutions at decreasing concentrations and erythrocyte filterability by filtration method. Different concentrations of aspirin, indomethacin and primaquine were prepared in absolute ethanol and incubated with red cells to measure their effects on fragility and deformability.**

Results: **Osmotic fragility curve was shifted to the left by the sickle red blood cells, the initial haemolysis occurred at concentration of 0.5% for normal and 4.5% for sickle cell. The concentrations of hypotonic saline that caused 50% lysis for the normal and sickle red cell were 0.41% and 0.33% respectively. The erythrocyte filtration time was 83.1± 11.4 seconds for normal and 106.4±23.9 seconds for sickle cell. Aspirin, indomethacin and primaquine produced no effect on osmotic fragility of both normal and sickle cells. Only primaquine at concentration of 2µg/ml caused a statistically significant prolongation in filtration time.**

Conclusion: **The sickle red blood cell resists in vitro haemolysis by hypotonic saline and has longer filtration time than the normal cell; primaquine has deleterious effect on the sickle cell in vitro. This drug should be given with caution to patients with sickle cell disease. Further studies to explore the in vivo effects are recommended.**

دراسة قابلية التحمل والتشكل للخلية المنجلية خارج الجسم: تأثيرات الأدوية المسكنة للألم والبريماكوين

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خلفية الدراسة: **أن خلية الدم الحمراء المنجلية تختلف من الناحية الفسلجية عن الخلية الطبيعية، كما أن تأثيرات الكثير من الأدوية، مثل المضادات الحياتية ومسكنات الألم سواء الضارة أو المفيدة على الخلية المتجلية غير معروفة وخاصة تلك ألتي لها قابلية على الأكسدة.**

الهدف: **أجريت الدراسة الحالية لتقصي مدى اختلاف الخلية المنجلية عن الخلية الطبيعية من حيث التحسس الأزموزي، والقابلية على التشكل، ودراسة تأثيرات الأدوية ذات التأثير المؤكسد المحتمل.**

طريقة البحث: **أخذت عينات دم من مرضى فقر الدم ألمنجلي (٣٩مريضا) ومن أشخاص متطوعين أصحاء (١٩) لمختلف**

أجراء الدراسة: **تم قياس الهيموغلوبين المنجلي بواسطة الترحيل الكهربائي، والتحمل الأزموزي بواسطة محلول كلوريد الصوديوم وبتراكيز متناقصة، كما تم قياس قابلية الترشيح عن طريق ترشيح الخلايا، و تم تحضير أدوية الأسبرين، الأندوميتاسين و البريماكوين بإذابة مسحوق الدواء بالكحول.**

النتائج: **أن كريات الدم المتجلية أدت إلى إزاحة منحنى التحلل إلى اليسار، حيث ظهر التحلل بتركيز ٠،٥% في الخلايا الطبيعية و٤،٥% للخلايا المنجلية. وكان تركيز المحلول الملحي الذي أدى إلى حدوث تحلل بنسبة ٥٠% هو ٠،٣٣% و ٠،٤١% للخلايا الطبيعية والمتجلية على التوالي وقد كان زمن الترشيح١،٨٣±٤،١١ ثانية و ١٠٦،٤± ٩،٢٣ للخلايا الطبيعية والمنجلية على التوالي لم يؤثر الأسبرين اوالأندوميثاسين أو البريماكوين على التحلل الأزموزي فيما أدى عقار البريماكوين فقط إلى إطالة زمن الترشيح. لم يؤثر أي من الأدوية على ظاهرة التمنجل خارج الجسم .**

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الاستنتاج: أن الخلية المنجلية تقاوم التحلل بواسطة المحلول الملحي أكثر من الخلية الطبيعية، كما أن لها زمن أطول للترشيح. إن لعقار البريماكوين تأثير ضار على الخلايا المنجلية خارج الجسم. وينصح باستخدام هذا العقار بحذر لهؤلاء المرضى كما نوصي بإجراء دراسات أخرى بإعطاء هذه الأدوية داخل الجسم .

INTRODUCTION

Haemoglobin S (HbS) differs from the normal haemoglobin (HbA) in the substitution of the amino acid valine for glutamic acid in the sixth position of the β-chain, resulting from DNA substitution of the nucleic base thiamine for adenine. This minor change in the structure is responsible for profound changes in the molecular stability and solubility of HbS.[1,2] At low oxygen tension, this mutant haemoglobin, polymerizes inside the red blood cell into gel and further into fibers or tactoids.[3] This polymerization is reversible when re-oxygenation occurs. However it becomes irreversible when cell membrane damage occurs leading to a drastic decrease in red cell deformability.[3,4] This leads to microvascular occlusion which may results in serious and sometimes fatal crises.[3] The sickling phenomena can be monitored directly by electron microscopy, or indirectly by measuring changes in blood viscosity or erythrocytes filterability.[1] The sickle erythrocytes are also sensitive to peroxidation more than normal erythrocytes. The peroxidative damage of the sickle erythrocytes may accelerate or contribute to the loss of cell deformability, the formation of irreversible sickle cells and chronic haemolysis.[5-8] Functional alteration of the cell membrane permeability to ions results in dehydration of the red cells, which leads to increase in the HbSS concentration within the cell, polymerization and irreversibility of the lesions.[9] The polymerization greatly impairs filterability of the sickle cell. The filterability of the sickle cell was found to be sensitive to small amount of intracellular polymer and that impairment of filtration was shown to be linearly related to polymer formation.[10] Red blood cells permeability and/or deformability are important for the maintenance of the erythrocyte shape and membrane integrity, both of which are impaired in sickle cells.[11] The sickle cell has high level of oxidized nicotinamide adenine dinucleotide phosphate level (NADP)[12] which leads to chronic redox imbalance. In addition, the sickle erythrocyte also has low level of reduced glutathione, which is an important antioxidant.[13,14] Sickle cell disease is frequently presents with complications as painful crises, infection and haemolysis which require intervention and frequent use of drugs. The effects whether useful or deleterious of various drugs as antibiotics and analgesics on the sickle red blood cell are not well known especially those with oxidative potential as primaquine, ciprofloxacin, sulphonamides and spirin. The present study is therefore intended to examine the difference in osmotic fragility and deformability between normal and sickle cells, and to study the effect of some of the frequently used drugs on both normal and the sickle cells in vitro.

PATIENTS, MATERIALS AND METHODS

Thirty eight patients (17 males and 21 females), with a mean age of (29±10) years, and 19 (5 male and 14 female) apparently healthy normal volunteers with a mean age of (31±8) years were included in various parts of this study. Patients with G6PD deficiency, those who had blood transfusion in the previous four months and those who were on medications apart from folic acid were excluded from the study, to avoid changes in HbS amount or the red cell characteristics. The study was approved by the College Ethical Committee and an informed written consent was obtained from patients and volunteers. Different numbers of patients or volunteers were selected for the various tests. The study was conducted in the department of Pharmacology, College of Medicine, University of Basrah, in the period between September, 2003 until September 2004. Cellulose acetate electrophoresis at alkaline pH was used to detect HbSS.[15] Screening for G6PD was done by fluorescent test.[9] Osmotic fragility test was carried out by exposing the red blood cells to decreasing concentrations of hypotonic sodium chloride solution and measuring the cell lysis by colorimetry.[15] Erythrocytes filterability was measured by recording the rate at which one milliliter of red cell suspension passed through a glass filter with pores of 10 µm diameter.[16]

Drugs were prepared by dissolving the pure powders of aspirin, indometacin and primaquine in absolute ethanol. This is used as stock solution and further dilutions to the required concentrations were made by the addition of phosphate buffer with pH adjusted to 7.4. The concentrations of drugs used were around their therapeutic serum levels following usual recommended therapeutic doses.[17] Sickling phenomenon was measured by slide method, in which a drop of blood mixed with the drug spread over slide covered with a cover slip and examined by light microscope after 24hours.[18] Statistical analysis was carried out using SPSS program version 11, unpaired –t test was used to evaluate the effect of drugs and to compare between the normal and sickle erythrocytes in both osmotic fragility and filterability. All tests were two-tailed and the level of probability taken as significant was 5% ($P<0.05$).

RESULTS

Osmotic fragility curve is shifted to the left by the sickle cells as shown in (Figure-1). The initial lysis of normal erythrocytes occurred at saline concentration of (0.5%), while for sickled erythrocytes it occurred at (0.45%) as shown in (Table-1 and Figure-1). The concentrations of saline which caused 50% lysis of normal and sickle cells were (0.41%) and (0.33%) respectively. The results showed that aspirin, indometacin and primaquine produced no significant decrease in osmotic fragility of normal erythrocytes. However these drugs had resulted in a small decrease in osmotic fragility of HbS erythrocytes which was statistically not significant, (Table-2). Comparison of filterability between HbS and HbA erythrocytes suspension is presented in (Table-3). There was a significant increase in time of filtration of sickled erythrocytes (106.4±23.9) seconds, as compared to normal erythrocytes (83.14±11.43) seconds. The effects of aspirin, indometacin and primaquine on filterability of normal and sickle erythrocytes are presented in (Table-3). Only primaquine (2µg/ml) resulted in statistically significant increase in filtration time. In normal erythrocytes, all drugs caused no significant changes in filtration time for sickle cells. All the drugs used in the present study had no significant effect on the percentage of in vitro sickling (the number of sickle cells in one microscopic field divided by the total number of red cells).

Table 1. The osmotic fragility of normal (HbA) and sickle (HbS) erythrocyte

NaCl %(mOsmol/L)	HbAA erythrocytes (n=12)	HbSS Erythrocytes (n=7)	T-value	Significance
0.9 (0.31)	0	0	-	-
0.6 (0.21)	0	0	-	-
0.55 (0.19)	0	0	-	-
0.5 (0.17)	7.5±5.12	0		
0.45 (0.15)	18.25±4.37	4.85 ± 3.11	7.10	0.0001
0.43 (0.14)	48.77±9.8	14.04±0.8	9.25	0.0001
0.4 (0.13)	9.14± 66.3	25.51±7.3	10.05	0.0001
0.35 (0.12)	88.24±7.81	38.95±8.66	12.76	0.0001
0.33 (0.11)	98.5±4.1	64.65±10.87	9.82	0.0001
0.3 (0.10)	± 0 100	83.42±7.0	-	-
0.1 (0.03)	100 ± 0	100±0	-	-

Data are presented as percentage of lysis (mean ± SD) of 12 normal subjects and 7 patients

Table 2. The effect of different drugs on osmotic fragility of erythrocytes with HbSS

***No significant differences between treatments P > 0.05(t-test)**

NaCl Concentration %(mOsmol/L)	% of lysis (mean±SD) of 7 patients				
	HbSS+no drug	HbSS + aspirin 500 µg/ml	HbSS+ Indometacin 5 µg/ml	HbSS+primaquine 2 µg/ml	Significant* difference
From 0.9 to 0.55 (0.31 to 0.19)	0	0	0	0	-
0.5(0.17)	0	0	0	0	-
0.45(0.15)	4.85±3.11	2.21±1.18	2.58±1.66	2.9±2.5	NS
0.43(0.14)	14.02±0.8	12.69±2.14	11.94±4.04	11.97±4.38	=
0.4(0.13)	25.51±7.3	20.69±4.48	23.17±9.89	21.72±10.76	=
0.35(0.12)	38.95±8.66	34.25±9.92	37.43±11.02	36.13±12.2	=
0.33(0.11)	64.65±10.87	58.27±6.8	58.38±8.61	58.98±17.23	=
0.3(0.1)	83.42±7.0	84.33±6.66	83.45±6.86	81.70±10.58	=
0.19(0.03)	100	100	100	100	=

Table 3. Filterability time in seconds of HbA and HbS erythrocytes with and without drugs (aspirin, indometacin and primaquine)

Sample	Filterability time (mean ± SD)	T-value
HbAA (normal)	83.14±11.43	
HbSS (no drug)	106.4±23.9*	2.32
HbSS Aspirin (500 µg/ml)	109.4±12.8*	0.43
HbSS Indometacin (5ug/ml)	117.1±11.3*	0.949
HbSS Primaquine (2ug/ml)	132.1±37.17**	2.828

***Significant difference from the normal group, (P< 0.05)**
**** Significant difference from the control group, (P< 0.05)**

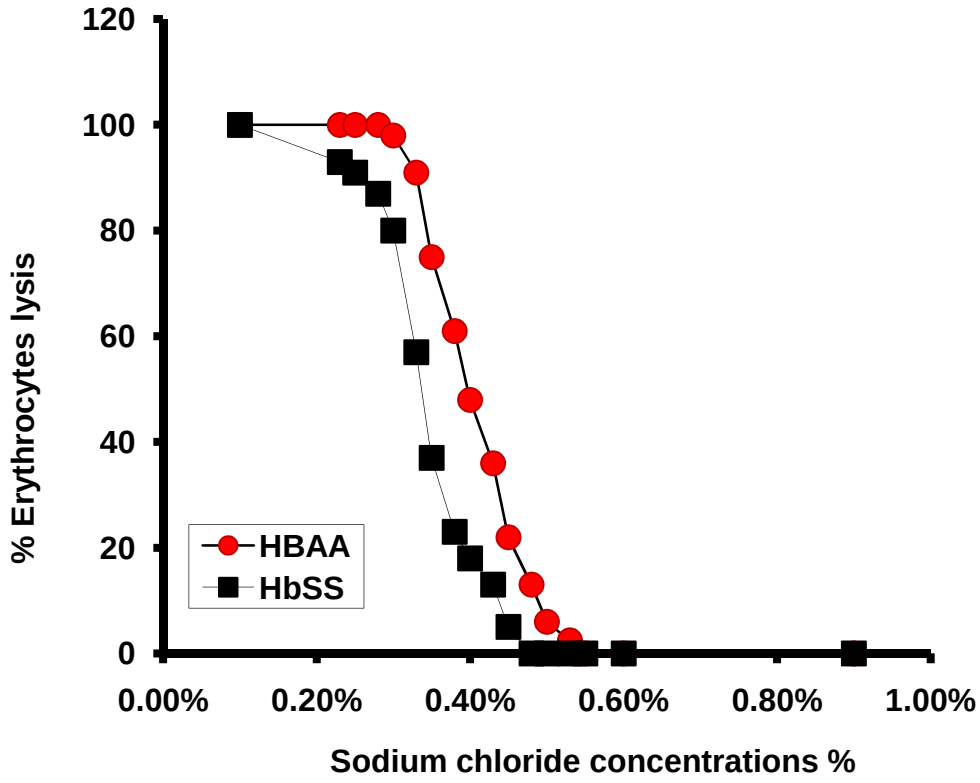

Fig1. Osmotic fragility of normal and sickle cells

DISCUSSION

We found in the present study that sickle erythrocytes resist haemolysis induced by hypotonic solution as compared to the normal red blood cell; a finding which is consistent with another study.[19] The decrease in osmotic fragility of sickle erythrocytes may be due to reduction in cell volume in relation to surface area due to polymer formation and cell dehydration due to activation of Ca^{2+} sensitive K^+ channels and $K^{\pm}C^-$ cotransporter of the red cell membrane.[20] The activation of the transporters is aggravated by the presence of irreversible sickle cells that show marked resistance to haemolysis due to high cell density and low water and ions content.[21] We also found significant increase in the filterability time of sickle erythrocytes in comparison to normal cells. This may be explained by presence of irreversible sickle cells that markedly decrease cellular deformability as a result of increased membrane rigidity caused by increased intracellular Ca^{2+} ions.[22,23] In the present study aspirin did not affect the in vitro sickling percentage. It was demonstrated that acetylation of sickle haemoglobin by aspirin is also pH dependent, as with increased pH, there was an increase in acetylation. The in vivo studies demonstrated that daily aspirin treatment leads to increased life span of the RBC probably due to acetylation of sickle haemoglobin.[24] Indometacin has no effect on in vitro sickling, however the in vivo effect may differ due to high production of various prostaglandins from the sickle cells.[25] It was found that prostaglandin E_2, PGI_2 and thromboxane levels are elevated in blood of patients with sickle cell disease, which contribute to pain during crisis, persistent vascular inflammatory process and increased liability to infection.[26,27] Of the different drugs used in the present study, primaquine was the only one that increases the time of filterability of the sickle cell. This is probably due to an increase in cell dehydration due to direct effect of the drug on the cell membrane that further aggravates the deoxygenation induced cell dehydration.[28] Indometacin also increases the filterability time, but this was not statistically significant. It is thus concluded that the sickle cell is more resistant to haemolysis than the normal red blood cell. Primaquine and probably indomethacin may have deleterious effect on the

sickle cell in vitro, although all these drugs have no effect on in vitro sickling. It is recommended that both drugs should carefully be administered to patients with sickle cell disease. Aspirin is less likely to cause such an effect. The in vivo effect of these drugs requires further investigation as the effect of drugs metabolites cannot be excluded.

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