INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 10, No. 1, p. 311-317, 2017

RESEARCH PAPER OPEN ACCESS

In vitro study of some characteristics of red blood cells with hemoglobin S

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Key words: Sickle cell, Osmotic resistance, Formation speed, Phenotype

http://dx.doi.org/10.12692/ijb/10.1.311-317 Article published on January 31, 2017

Abstract

Sickle cell anemia is hemoglobin sis due to mutated S hemoglobin widespread in the black race. The aim of this work was to determine some biological properties of erythrocytes with hemoglobin S in order to monitor the efficacy of anti-sickle cell traditional treatments. 393 patients were taken from the Zou regional hospital in Benin for hemoglobin electrophoresis, the Emmel test and the osmotic resistance of red blood cells. The hemoglobin electrophoresis phenotyped 63.87% AA, 17.81% AS, 10.94% AC, 2.80% SS, 4.07% SC and 0.51% CC. The Emmel test was positive in 100% of SS cases, 90% of AS cases and 93.75% of SC cases. The rate of sickle cell formation was less than one hour in 100% SS and 72.73% SC. In the AS phenotype, the appearance of sickle-cell was rather progressive (25% of cases in one hour, 35% in two hours, 20% in three hours and 20% in four hours) suggesting competition between both types of hemoglobin. Compared to the AA phenotype, the osmotic resistance of the red cells increased significantly in the AS phenotype and very significantly in the SS and SC phenotypes, indicating an increased erythropoiesis compensating the sickle cell hemolysis. Hemoglobin S was associated with an excellent osmotic resistance of red blood cells and the rate of sickle cell formation depended on the hemoglobin phenotype. These two parameters can be used to monitor the efficacy of traditional remedies proposed to treat sickle cell anemia.

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Introduction

Sickle cell disease, also known as sickle cell anemia, is a severe hemoglobin opathy commonly encountered in the black race. Clinically, it is manifested by a chronic hemolytic anemia (Essono Mvo a *et al.*, 2004). It is the most common and severe form of hemolytic anemia due to a structural abnormality of hemoglobin (Lab i e *et al.*, 1984). Its diagnosis is made by several tests but the electrophoresis of the hemoglobin in alkaline medium constitutes the technique commonly used in clinical biology. However, this method does not separate hemoglobin's S and D, hence the need for complementary techniques to differentiate these two he mo globins (Segbena *et al.*, 2002; Bardakjian-Mich au *et al.*, 2003).

Two types of hemoglobin sis are common in black Africa. These are the S and C. These hemoglobin opathies are caused by point mutations on the β chain of normal hemoglobin A in position 6. Glutamic acid is replaced by valine in hemoglobin S and lysine in hemoglobin C. Both mutations offer protection against malaria (Modiano, 2001; Benno Kreuels, 2010; Piel, 2013; Williams, 2016). The mechanism of this protection would imply an alteration of the plasmodium traffic in the red blood cell by the inhibition of the polymerization of the cell act in (Cyrklaff, 2011).

Various treatments are available to treat or prevent sickle cell anemia. Phototherapy or treatment of diseases by medicinal plants is very encouraged by the World Health Organization (WHO) in our developing countries with very poor populations (Jorim, 2012; Khan, 2013; Hughes, 2013). Testing the therapeutic efficacy of proposed plants to treat sickle cell disease requires prior control of the biological parameters of the disease.

Previous studies investigated the effect of *Justicia secunda* Vahl. extract on the solubility of haemoglobin S and membrane stability of sickle erythrocytes (Mpiana *et al.*, 2010), antisickling property of *Scoparia dulcis* Linn. leaves (Abere *et al.*, 2015) and anti-sickling activity of *Ocimum gratissimum* L. leaves extract (Tshilanda *et al.*, 2015).

This work aimed to determine *in vitro* some properties of the red blood cells with hemoglobin S in order to monitor the efficacy of traditional remedies proposed to treat sickle cell anemia.

Material and methods

Biological material

Blood was collected on EDTA in 393 consenting patients at the Zou departmental hospital in Benin from March to June 2013. In these samples, hemoglobin electrophoresis, the Emmel test and the osmotic resistance of the red blood cells were performed.

Electrophoresis of hemoglobin

- Principle: It is based on the differential migration of hemoglobin's in an electro phoretic field according to their electrical charges.
- Preparation of the hemolysate: Centrifuge at 3000 rpm of the blood taken on anticoagulant for five minutes. The plasma and the float leucocytes were removed and then the red cell pellet was washed three times with physiological water at 3000 rpm for 5 minutes. In a tube, a drop of globular pellet was hemolysed with six drops of the hemolyzing reagent (1% sapronin solution).

Technique:

The two compartments of the migration tank were filled to the mark with the Tris-Glycine buffer ph = 8.4. The cellulose acetate membrane was soaked for at least fifteen minutes. The humiliates were deposited in the wells of the sample vessel carrier. The membrane was then dried slightly between two sheets of blotting paper to remove the excess buffer and then placed on the rack of the migration tank (absorbing side up). Using an applicator, the humiliates were removed and deposited on the cathode-side (-) of the membrane. The migration was made for one hour and thirty minutes at 200 volts, 5mA.

- Preparation of Tris-Glycine Buffer pH = 8.4
• Trishy droxy methylamino methane 10.2g
• EDTA 0.6 g
• Boric acid 3.2g
• Distilled water QSP 1000ml

Emmel test

- Principle: In the absence of oxygen hemoglobin S polymerizes resulting in the formation of fibers that distort the blood cell and gives it a sickle aspect.
- Technique: One drop of blood was deposited on a slide. A drop of 2% sodium metabi sulfite was added. Both drops were carefully mixed and the preparation was then covered without air bubble. The contours were sealed with a candle to prevent desiccation. The result was read the next day. The test was positive if the erythrocytes took a form of sickle.

Sickle cell formation speed

- Principle: the time taken by the red blood cell to turn into sickle.
- Technique: The Emmel test was read every 30 minutes for 4 hours and then the next day.

Osmotic resistance test

- Principle: It was based on the ability of erythrocytes to resist to haemolysis in a hypotonic solution.
- Technique: Blood was diluted 1/200 in two salt solutions of different concentrations. One was isotonic (0.9% Na C l) and the other hypotonic (0.54% Na C l). Red cells were counted with a Malassez cell. The ratio of the number of red blood cells counted in the hypotonic solution over that of the isotonic solution was the percentage of red blood cells resistant to hemolysis.

Statistical analysis

Graphs were plotted using Graph pad software. In each group, the different means were compared to that of Hemoglobin AA using ANOVA one way, Dennett's Multiple Comparison Test. The significance level was set at 5%.

Results

The phenotypic distribution of hemoglobin in the study population

The results of the hemoglobin electrophoresis are shown in Table 1.

Of the 393 patients collected, 251 were AA phenotype and represents 63.87% of the study. 70 patients were heterozygous AS, accounting for 17.81% of the samples. 43 were heterozygous AC phenotype and account for 10.94% of the cases. 11 were homozygous SS and represent 2.80% of the total number of patients. 16 had a heterozygous SC phenotype and made 4.07%. Only 2 patients were CC homozygous and made 0.51% of the study.

The Emmel test was not positive in all carriers of sickle cell trait

The test was performed on samples having hemoglobin S. 97 AS, SS and SC phenotypes were diagnosed by electrophoresis. The result of the Emmel test was shown in Table 2.

Table 1. Frequencies of different types of hemoglobin.

Hemoglobin	Number	Frequency (%)
AA	251	63.87
AS	70	17.81
AC	43	10.94
SS	11	2.80
SC	16	4.07
CC	2	0.51
Total	393	100.00

Table 2. Détection of sicklecell.

Emmel test	Number		Proportion en %		Total	
	Positive	Negative	Positive	Negative	Number	Proportion
AS	63	7	90	10	70	100
SS	11	0	100	0	11	100
SC	15	1	93.75	6.25	16	100
Total	89	8	91.75	8.25	97	100.00

In general, sickle-cells were detected in 89 of the 97 samples, indicating 91.75% positivity of Emmel test in all cases. 8 out of 97 samples remained negative. Of the 70 AS samples analyzed, the Emmel test was positive in 63 cases, representing 90% of cases.

The test is positive in all 11 SS cases analyzed. In SC cases, we observed the presence of sickle cells in 15 samples out of 16 samples analyzed, I e 93.75% of the cases.

The rate of sickle cell appearance depended on the hemoglobin phenotype

42 samples were analyzed divided into 20 AS cases, 11 SS cases and 11 SC cases. Their distribution according to the time of onset of sickle cell was recorded in Table 3.

Table 3. Proportion of samples positive for the Emmel test over time.

Time (h) / Hemoglobin	1		2		3		4		Total	
	N	P	N	P	N	P	N	P	N	P
AS	05	25.00	7	35.00	4	20.00	4	20.0	20	100
SS	11	100.0	0	00.00	0	00.00	0	0.00	11	100
SC	08	72.73	0	00.00	3	27.27	0	0.00	11	100
Total	24	57.14	7	16.67	7	16.67	4	9.52	42	100

E: Number; P: Proportion; (H): (hour).

In the AS samples, the test was positive in 5 cases (25%) at the first hour, 7 other cases (35%) at the second hour, 4 new cases (20%) at the third hour, and In the last 4 cases (20%) at the fourth hour. In SS samples, the test was positive in all cases from the first hour. As for the SC, the Emmel test was positive in 8 cases (72.73%) from the first hour and in the 3 remaining cases (27.27%) in the third hour.

Hemoglobin S was associated with excellent osmotic resistance of erythrocytes

The percentage of red blood cells resistant to hemolysis in a hypotonic solution (0.54% Na C l) was determined for the different hemoglobin phenotypes. The results were shown in Fig. 1.

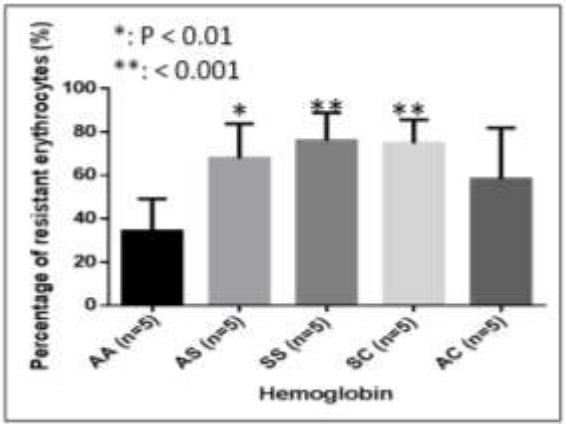

Fig. 1. Osmotic resistance of different types of hemoglobin.

At the concentration of 0.54% Na C l, more than half of the AA cells are hemolyzed. The percentage of resistant erythrocytes is on average 34 ± 15%. The percentage of resistant erythrocytes significantly higher is above average in the other phenotypes. It is 68 ± 16% for the AS phenotype; 76 ± 13% for the SS phenotype; 75 ± 11% for the SC phenotype and 58 ± 10 % for the AC phenotype. Compared to the normal AA phenotype, the increased percentage of resistant erythrocytes were significant in the AS phenotype (P <0.1) and highly significant in the SS and SC phenotypes (P <0.01). The increase osmotic resistance in the AC phenotype was not significant.

Discussion

Sickle cell anemia is a hemoglobin opathy widespread in the black race (Pagnier *et al*, 1984; Segbena *et al*, 2002). To test the efficacy of traditional medicines proposed to alleviate sickle cell crises, a control of biological parameters of disease is required (Abere *et al*, 2015; Tshilanda *et al*, 2015; Amujoyegbe *et al*, 2016). For this purpose, this work was devoted to the study of some parameters such as the rate of sickle cell formation and the osmotic resistance of red blood cells with hemoglobin S. To achieve this, phenotyping by electrophoresis of hemoglobin was first performed.

It revealed 63.87% AA subjects, 17.41% AS subjects, 10.94% AC subjects, 2.80% SS subjects, 4.07% SC subjects, and 0.51% subjects CC in the patient population received at the Zou Departmental Hospital in Benin. This phenotypic distribution confirms that previously found in the same department by other authors (Chippaux *et al.*, 1992).

The Emmel test was positive in 100% SS cases, 94% of SC cases and 90% of AS cases. Hemoglobin D migrates at the same rate as hemoglobin S. It is unlikely that we have cases of hemoglobin D in our samples because this hemoglobin does not form sickle cell and moreover it is especially characteristic of Asian regions (Bardakjian-Michau, 2003; Oliver, 2008; Aubry *et al.*, 2012).

To better study the characteristics of hemoglobin S, we then evaluated the rate of appearance of sickle-cell anemia in the various phenotypes of S. After one hour, the Emmel test was positive in all SS cases and in 72.73%, or about three-quarters of SC. The test was only positive in 25% of the AS, which demonstrates the protective effect of hemoglobin A in the red blood cell of AS heterozygotes. This result was confirmed at three hours when the test was positive in all SC cases while there was still 20% of AS cases negative. Hemoglobin C did not provide the same protection to heterozygous SC erythrocytes as hemoglobin A to AS. This was not surprising because hemoglobin C is after all abnormal hemoglobin and the individual SC is also considered having the sickle cell disease (Rees, 2010; Aubry *et al.*, 2012).

The time of onset of sickle cell disease increased progressively in the AS phenotype over time: 25% positive after one hour, 60% positive after two hours, 80% after three hours and 100% after four hours. Such an evolution could be explained by a quantitative variation of hemoglobin A from one individual AS to another. Sickle cell formation is due to the polymerization of hemoglobin S in hypoxic medium. It has been pointed out that the time required for polymerization (delay time) also depends on the content of hemoglobin F which inhibits polymerization when it reaches 20%. (Bardakjian-Michau, 2003, Oliver, 2008).

All the Emmel test were positive after four hours. It is therefore essential to wait at least four hours of reading before declaring a negative test performed by this method. When we determined the osmotic resistance of erythrocytes in 0.54% Na Cl solution, the percentage of hemolysis resistant erythrocytes was only 34 ± 15% in the normal AA phenotype, less than half the cases. The percentage of resistance in the AS was 68 ± 16%. It was significantly higher than that of the AA phenotype (P value <0.01). This high osmotic resistance reflected a high proportion of young red blood cells, as a result of hematopoiesis compensating for hemolytic anemia characteristic of hemoglobin S (Benno Kreuels, 2010). Indeed, the increase of reticulocytes (young red blood cells) has been demonstrated in the blood of individuals with hemoglobin S (Meier, 2013).

In SS and SC phenotypes, hemolytic anemia was more severe than in the AS phenotype, hematopoietic blood cell regeneration was deeply increased. The preponderance of young erythrocytes in these samples could explain the extremely significant increase in osmotic resistance in these groups compared to the normal AA phenotype (76 ± 13% and 75 ± 11% respectively, P value <0.001). The osmotic resistance of 58 ± 10, 23% in the AC phenotype was also increased relative to AA. However, the increase was not statistically significant. This phenomenon could be explained by the micro cytosis characteristic of this phenotype (Orphaned, 2013) and not an increased production of young red cells as in the AS case. Indeed, even in CC homozygotes erythropoietin was altered (Wickramasinghe, 1982).

Conclusion

Sickle cell hemoglobin S was associated with excellent cellular osmotic resistance most likely due to the youthfulness of the erythrocytes resulting from increased erythropoiesis. The sickle cells forming speed varies depending on the hemoglobin phenotype and would function in AS heterozygotes relative to the quantitative relationship between hemoglobin A, F and S in the red cells. Osmotic resistance and sickle formation speed can serve as a benchmark in monitoring the effectiveness of traditional remedies proposed to treat sickle cell disease.

References

Abere TA, Okoye CJ, Agoreyo FO, Eze G, Jesuorobo RI, Egharevba CO, Aimator PO. 2015. Antisickling and toxicological evaluation of the leaves of *Scoparia dulcis* Linn (Scrophulariaceae).

BMC Complementary and Alternative Medicine 15, 414.
DOI: 10.1186/s12906-015-0928-5.

Amujoyegbe OO, Idu M, Agbedahunsi JM, Erhabor JO. 2016. Ethnomedicinal survey of medicinal plants used in the management of sickle cell disorder in Southern Nigeria. Journal of Ethnopharmacology **185:** 347-60.
DOI: 10.1016/j. jep.03.042.

Aubry P et Gauzere B-A, 2016. Hémoglobinoses, Medécine tropicale.
www.google.fr/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&cad=rja&uact=8&ved=0ahUKEwidioPozq_RAhXIwBQKHTWhBmUQFgggMAE&url=http%3A%2F%2Fmedecinetropicale.free.fr%2Fcours%2Fhemoglobinoses.pdf&usg=AFQjCNHPw2pAvdtZ5fm0VlCmEmpMPRtbmA&bvm=bv.142059868,d.d24.

Bardakdjian-Michau J, Dhondt JL, Ducrocq R, Galactéros F, Guyard A, Huchet FX, Lahary A, Lena-Russo D, Maboudou P, North ML, Prehu C, Soummer AM, Verschelde M, Wajcman H. 2003. Bonnes pratiques de l'étude de l'hémoglobine. Annale de Biologie Clinique **6,** 401-409.

Benno K, Christina K, Robin K, Matilda A-A, Peter A-T, Benedicta T, Christa E, Samuel A, Iris L, Ohene A, Jurgen. 2010. Differing effects of HbS and HbC traits on uncomplicated *Falciparum malaria*, anemia, and child growth. Blood **115(22),** 4551-4558.

Chippaux J-P, Massougbodji A, Castelw J, Akogbeto M, Zohoun I, Zohoun T. 1992.Parasitémies à Plasmodium falciparum ou P. malariae chez les porteurs du trait drépanocytaire dans différents biotopes du Bénin. Revue d'Epidémiologie et Santé Publique **40,** 246-251.

Cyrklaff M, Sanchez CP, Kilian N, Bisseye C, Simpore J, Frischknecht F, Lanzer M. 2011. Hemoglobins S and C interfere with actin remodeling in *Plasmodium falciparum* infected erythrocytes. Science **334 (6060),** 1283-6.

Essono Mvoa E, Nkoa T. 2004. Diagnostic et anomalies biologiques chez un drépanocytaire. Clinics in Mother and Child Health **1(1),** 12, 13.

Hughes GD, Aboyade OM, Clark BL, Puoane TR. 2013. The prevalence of traditional herbal medicine use among hypertensives living in South African communities. BMC Complementary and Alternative Medicine **18,** 13, 38.

Jorim RY, Korape S, Legu W, Koch M, Barrows LR, Matainaho TK, Rai PP. 2012. An ethnobotanical survey of medicinal plants used in the eastern highlands of Papua New Guinea. Journal of Ethnobiology and Ethnomedecine **18,** 8,47.

Khan SM, Page S, Ahmad H, Shaheen H, Ullah Z, Ahmad M, Harper DM. 2013. Medicinal flora and ethnoecological knowledge in the Naran Valley, Western Himalaya, Pakistan. Journal of Ethnobiology and Ethnomedecine **10,** 9:4.

Labie D, Wajcman H. 1984. Biologie de l'hémoglobine S: Epidémiologie et génétique; physiopathologie; biologie clinique; diagnostic anténatal, In: La maladie drépanocytaire, Sandoz editions 14Ŕ 63.

Modiano D, Luoni G, Sirima BS. 2001. Hemoglobin C protects against clinical *Plasmodium falciparum* malaria. Nature **414,** 305-308.

Mpiana PT, Ngbolua KNN, Bokota MT, Kasonga TK, Atibu EK, Tshibangu DST, Mudogo V. 2010. *In vitro* effects of anthocyanin extracts from *Justicia secunda* Vahl. on the solubility of haemoglobin S and membrane stability of sickle erythrocytes. Blood transfusion **8,** 248-54
DOI 10.2450/2009.0120-09.

Oliver M. 2008. Hémoglobinopathies. Diagnostic au laboratoire, pièges de l'interprétation. Expérience del' Hôpital d'Instruction des Armées Laveran. Feuillets de Biologie **49,** 5-13.

Pagnier J, Mears JG, Dunda-Belkhodja O, Schaefer-Rego KE, Beldjord C, Nagel RL, Labie D. 1984 Evidence for the multicentric origin of the sickle cell hemoglobin gene in Africa. Proceedings of the National Academy of Sciences of the United States of America **81,** 1771-1773.

Piel FB. 2013. Sickle-cell disease: geographical distribution and population estimates. Medecine/Sciences **29,** 965-7.

Rees DC, Williams TN, Gladwin MT. 2010. Sickle disease. The Lancet **376,** 2018-2031.

Segbena AY, Kueviakoe I, Messie AK, Napo-Koura IG, Vovor A, David M. 2002. Hemoglobin nomalies at the university hospital center in Lome, Togo. Médecine tropicale: revue du Corps de santé colonial 62(1), 51-4.

Tshilanda DD, Onyamboko DN, Babady-Bila P, Ngbolua KT, Tshibangu DS, Dia FitaDibwe E, Mpiana PT. 2015. Anti-sickling Activity of Ursolic Acid Isolated from the Leaves of *Ocimum gratissimum* L. (Lamiaceae). Natural Products and Bioprospecting **5(4),** 215-21.
DOI: 10.1007/s13659-015-0070-6.

Wickramasinghe SN, Akinyanju OO, Hughes M. 1982. Dyserythropoiesis in homozygous haemoglobin C disease.Clinical & Laboratory Haematology **4(4),** 373-81.

Williams TN. Sickle cell disease in sub-Saharan Africa. Hematology/Oncology Clinics of North America **30(2),** 343-358c.