Phase angle correlates with n-3 fatty acids and cholesterol in red cells of Nigerian children with sickle cell disease

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Abstract

Objective: To determine the cholesterol content and fatty acid composition of red cell membrane phospholipids (PL) of children with sickle cell disease (SCD) and to correlate these levels with whole body phase angle that is related to the integrity and function of cell membranes.

Study design: Blood samples were obtained from 69 children with SCD and 72 healthy age- and gender-matched controls in Nigeria for the determination of the cholesterol content and proportions of fatty acids in red cell PL. Bioelectric impedance analysis was used to obtain resistance (R) and reactance (Xc) from which phase angle was calculated as arctan Xc/R. Cholesterol (normalized to lipid phosphorus) and the proportions of individual fatty acids were correlated with phase angle.

Results: The proportions of palmitic (p < 0.001), stearic acid (p = 0.003) and cholesterol (p < 0.001) were significantly higher in the red cells of children with SCD, whereas the proportions of arachidonic acid and docosahexaenoic acid were reduced (p = 0.03 and < 0.001, respectively) compared to controls. The phase angle was inversely correlated with the proportions of palmitic acid (p = 0.03) and oleic acid (p < 0.001) and cholesterol (p = 0.003). Three n-3 polyunsaturated fatty acids-eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid-were positively correlated with phase angle (p < 0.001).

Conclusions: The fatty acid composition and cholesterol content of tissue membranes in SCD correlate with the phase shift measured by bioelectric impedance analysis. Phase angle measurements may provide a non-invasive method for monitoring interventions aimed at altering the lipid composition of membranes.

Introduction

Sickle cell disease (SCD) is the most common genetic disorder in African and African American populations and it is associated with increased morbidity and mortality in
affected individuals [1]. Children with SCD exhibit impaired growth [2–4] as well as delayed skeletal and sexual maturation [5]. The underlying cause of growth retardation in SCD has not been confirmed but has been attributed to several factors such as increased resting metabolic rate [6] and deficiencies of various nutrient including folate, zinc, vitamin A, vitamin E and iron [7].

In a previous study of the fatty acid composition of the serum phospholipids of children with SCD and healthy controls that we conducted in Nigeria [8], we found that the serum phospholipids of SCD patients and controls had comparable levels of linoleic and α-linolenic acid. However, the percentages of the long-chain polyunsaturated fatty acids derived from these two essential fatty acids were significantly decreased whereas the proportions of saturated fatty acids, namely oleic acid and palmitic acid, were significantly increased. Similar alterations in the serum total phospholipid composition of children with SCD have been reported by others [9].

The fatty acid composition of serum phospholipids is known to reflect the phospholipid composition of cell membranes [10]. Because the fatty acid composition of membrane phospholipids is a major determinant of membrane fluidity and function [11], any alteration in fatty acid composition of membrane phospholipids could contribute to the red cell abnormalities seen in SCD, such as cation imbalance, dehydration, reduced deformability and hypercoagulability [12,13].

Phase angle is a bioelectrical impedance parameter derived from the measured impedance parameters, resistance (R) and reactance (Xc). Whereas R is related to the magnitude of the lean body mass, Xc reflects the capacitance produced by cell membranes and tissue interfaces. This capacitance causes the current to lag the voltage, creating a phase shift. Phase angle is regarded as an indicator of cellular health and membrane integrity, a low phase angle being indicative of a deterioration in the integrity or function of cell membranes. Phase angle has been shown to be a reliable predictor of outcome in a variety of clinical conditions where alterations in cell membranes are known to occur, including sepsis [14], trauma [15], HIV infection [16] and cancer [17].

In a recent study of Nigerian children with SCD, we determined the fatty acid composition of serum phospholipids and correlated fatty acid proportions with the phase angle [18]. The percentage of palmitic acid and oleic acid correlated inversely with phase angle, whereas, in contrast, three polyunsaturated fatty acids of the n-3 series (eicosapentaenoic, 20:5n-3; docosapentaenoic, 22:5n-3; and docosahexaenoic, 22:6n-3) were positively correlated with phase angle. If serum phospholipids are truly surrogates for membrane phospholipids, as is widely believed, then the correlations we observed between specific fatty acids and phase angle should apply to the fatty acids of tissue membrane phospholipids as well.

Cholesterol and phospholipids are the major lipid components of cell membranes and the major determinants of membrane fluidity and function [11]. In the present study, using the cholesterol content and the fatty acid composition of red cell membranes as surrogates for tissue membranes in children with SCD and healthy controls, we found significant correlations between phase angle with both cholesterol and the proportions of specific n-3 polyunsaturated fatty acids in the red cell phospholipids of these subjects.

**Methods**

Subjects with sickle cell disease (33 males and 36 females) were recruited from among the patients at the pediatric clinics at both the Jos University Teaching Hospital (JUTH) and Evangel Hospital in Jos, Nigeria. The SS genotype of the SCD subjects was confirmed by cellulose acetate electrophoresis of red blood cell lysates. Age-and gender-matched controls (36 males and 36 females) were recruited from among children visiting the clinic for follow up examinations or from among the children of the staff of JUTH. This study was approved by the Ethics Review Committee of JUTH and by the Human Research Review Committee of the University of New Mexico School Health Sciences Center, Albuquerque, NM.

**Sample collection**

Blood was obtained by venipuncture and collected into vacutainer tubes containing EDTA as the anticoagulant. After centrifugation at 5,000 × g for 10 min, the plasma fraction was removed. The red blood cells were washed twice with 0.9% (w/v) NaCl to remove plasma, platelets and leukocytes and then resuspended in saline. The red blood cell suspension was then aliquoted into cryovials and frozen at -40°C until transported in the frozen state to the University of New Mexico School of Medicine for analysis.

**Fatty acid analysis of red cell phospholipids**

Briefly, the total lipids in 0.15 ml of red blood cells were extracted into chloroform: methanol (1:1, v/v). The extract was evaporated under a stream of nitrogen, redissolved in 0.5 ml chloroform, and loaded onto a silicic acid column which had previously been prepared and washed with chloroform. Neutral lipids were eluted with 5 ml chloroform. Total phospholipids were eluted with 6 ml chloroform: methanol (2:1, v/v). The eluate was again dried under a stream of nitrogen and redissolved in 0.5 ml 15% (w/v) BF₃ in methanol. Preparation of fatty acid methyl esters was carried out as previously described by

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Morrison and Smith [19]. The methylation reaction was carried out at 100°C for 10 min. Fatty acid methyl esters were quantitated using a 0.53 mm × 15 m fused silica Megabore DB-225 column (J & W, Folsom, CA) in a gas chromatograph (Hewlett Packard, model 5890) equipped with an integrator. Fatty acids were identified by comparison of their retention times to those of fatty acid standards (Supelco, Inc., Bellefonte, PA) and results were expressed as weight percent of total fatty acids.

**Cholesterol analysis of red cell membrane**

Cholesterol was extracted from the red cell suspension as follows. One ml of red cell suspension was extracted with 30 ml of chloroform/methanol (2:1, v/v) and the total lipids reconstituted in 0.5 ml chloroform. To a 200 µl aliquot of the total lipid extract 1 ml ethanol and 300 µl potassium hydroxide solution (30% by weight) were added. The mixture also contained 107 µg of 5-α-cholestane (internal standard). The mixture was heated at 80°C for 20 minutes. Following saponification, 1 ml water and 2 ml hexane were added to the mixture. After mixing and centrifugation, the upper hexane layer was removed and transferred to a clean test tube and the aqueous layer was extracted twice more with 1.5 ml hexane. The combined hexane layers were evaporated to dryness under a stream of nitrogen and the extracted cholesterol was dissolved in hexane for gas chromatographic analysis.

Cholesterol was analyzed using a Hewlett-Packard gas liquid chromatograph (model 5890) equipped with a flame ionization detector and a fused-silica capillary column (SAC-5, 30 m × 0.25 mm, Supelco, Bellefonte, PA) and helium carrier gas. The injector and detector temperature were maintained for 15 min and then increased to 290°C over 5 min and held at 290°C for 5 min. Cholesterol content was expressed as mg/ml red cell suspension volume and then normalized to phosphorus.

**Determination of red cell phosphorus**

All glassware used for phosphorus analysis was rinsed with hydrochloric acid and deionized water. One ml of washed red blood cells was extracted with 20 ml of chloroform:methanol (2:1, v/v) for one hour at room temperature. After addition of 4 ml of saline solution, the mixture was vortexed and centrifuged at 1,000 rpm for 5 min to obtain two phases. The chloroform layer was filtered and then evaporated under a stream of nitrogen.

Concentrated sulfuric acid (0.5 ml) was added to the tubes containing the extracted phospholipids and the samples were heated in a sand bath for 3 hours at 250°C. After the addition of perchloric acid (0.15 ml, 70%), the samples were heated at 250°C for an additional 30 min, mixing occasionally. After cooling, 9.1 ml of an aqueous ammonium molybdate solution (26% by weight) and 0.4 ml of Fiske-Subbarow reagent were added and the samples were then placed in a boiling water bath for 10 min. The absorbance at 820 nm was measured and the phosphorus concentration determined using a phosphorus standard curve constructed using disodium hydrogen phosphate (equivalent to 0.5 to 20.0 µg of phosphorus).

**Bioelectrical impedance analysis**

Impedance analysis was performed using a BIA-Quantum impedance analyzer (RJL Inc, Clinton Township, MI). The instrument delivers an alternating 800 µamp current at a frequency of 50 kHz. Subjects were requested to void before measurements were made. The subjects laid in the supine position on a nonconducting surface with limbs abducted approximately 30° from the body. Signal-introducing electrodes were placed on the first joint of the middle finger and just below the middle toe. The detecting electrodes were placed with the upper edge of the electrode bisecting the ulnar head of the wrist and the median malleolus of the foot. Care was taken to ensure that the signal and detecting electrodes were not closer than 5 cm. Duplicate measurements of resistance (R) and reactance (Xc) were made on each subject. Phase angle was calculated as the arctan Xc/R and is expressed in degrees.

**Calculation of mean melting point (MMP) of red cell membrane phospholipid**

The MMP was determined by as described by Jensen and Patton [20]. First, the mol% of each fatty acid was calculated by dividing the mass% of each fatty acid by its respective molecular weight. Next, we multiplied the mol% by the (MP +100) of each fatty acid to obtain the melting point fractions. Finally, the MP fractions were summed and 100 was subtracted from that sum to provide the estimate of the MMP of the acyl chains of the phospholipid preparation. The melting points used for the fatty acids are as follows: C14:0 (54°C), C15:0(52°C), C16:0 (63°C), C16:1 (-5°C), C18:0 (69°C), C18:1n-9 (16°C), C18:1n-7,(44°C), C18:2n-6 (-5°C), C18:3n-6 (-11.3°C), C18:3n-3 (-10°C), C20:4n-6 (-49°C), C20:5n-3 (-54°C), C22:5n-3 (-54°C), C22:6n-3 (-44.1°C), C22:6n-3 (-44°C).

**Statistical analysis**

Statistical analysis was performed with the aid of NCSS statistical software (NCSS, Kaysville, UT). The phospholipid fatty acid proportions and the cholesterol content of the red cells of SCD subjects and controls were compared using a two-sample t-test. Correlations of the various fatty acid percentages with phase angle and MMPs were estimated using regression analysis. A p-value < 0.05 was considered significant.
Results

Comments on the study population
As shown in Table 1, the mean age of the male SCD subjects was not significantly different than the mean age of the male controls. The female SCD subjects were also matched for age with the controls. No significant differences in any of the body composition parameters such as weight, height, %FFM or %BF were observed between either the male or female subjects and their respective controls. The only exception was the phase angle for which there were significant differences between controls and SCD subjects for both males and females (Table 1).

Fatty acid profiles of red cell phospholipids
Because no differences were found in the fatty acid proportions between male and female controls and between male and female SCD subjects, the fatty acid data for male and female control subjects were pooled and the data for male and female SCD subjects were combined. As shown in Table 2, the red cell phospholipids of the SCD subjects contained significantly higher proportions of the two major saturated fatty acids, palmitic acid (C16:0) and stearic acid (C18:0), than the controls. The monounsaturated fatty acids oleic acid (18:1n-9) and vaccenic acid (18:1n-7) were also significantly elevated in the red cell membrane phospholipids of the SCD subjects.

With regard to the n-6 family of polyunsaturated fatty acids, only α-linoleic acid (C18:2n-6) and arachidonic acid (C20:4n-6) were significantly reduced in the membrane phospholipids of the SCD subjects compared to controls. For the n-3 family of polyunsaturated fatty acids, the long chain polyunsaturated fatty acids derived from α-linolenic acid (C18:3n-3), namely eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3), were significantly reduced in the membrane phospholipids of the SCD subjects. Overall, the SCD subjects had increased proportions of saturated fatty acids in their red cell phospholipids and significantly decreased proportions of polyunsaturated fatty acids (Table 2).

The Mean Melting Point (MMP) of red cell phospholipids
The finding of higher proportions of the high melting saturated fatty acids and lesser amounts of the PUFA with lower melting points in the red cell phospholipids of the SCD subjects relative to the controls prompted us to assess the overall impact of these differences on the relative fluidity of the acyl chains of the phospholipids from the red cells of the SCD patients and the controls by estimating the MMP of the phospholipids using the approach of Jensen and Patton [20]. The calculated MMP of the SCD subjects was significantly higher than for the controls, 29°C vs 24°C, p < 0.001. The estimated MMP of the red cell phospholipids was also inversely correlated with phase angle as shown in Figure 1 (p = 0.001).

Correlation of phase angle with individual phospholipid fatty acids and cholesterol
Of the major saturated fatty acids that were found to be significantly different between the phospholipids of the SCD patients and controls, only palmitic acid exhibited a significant inverse correlation with phase angle (p = 0.03). A negative correlation with phase angle was also seen with oleic acid, the major monounsaturated fatty acid in the red cell phospholipids (Fig. 2, p < 0.001.). Of the two nutritionally essential fatty acids, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), only linoleic acid was significantly correlated with phase angle (p = 0.012).

No significant relations were found between phase angle and the long-chain polyunsaturated fatty acids of the n-6 series. In contrast, significant positive correlations were found between the phase angle and the following long-chain polyunsaturated fatty acids of the n-3 series and

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Table 1: Summary of the characteristics of the controls and the subjects with SCD

|                  | Males SCD | Males Controls | P-value | Females SCD | Females Control | P-value |
|------------------|-----------|---------------|---------|-------------|----------------|---------|
| Age (yrs)        | 10.3 ± 4.8| 10.6 ± 5.4    | NS      | 11.8 ± 4.9  | 11.7 ± 4.8     | NS      |
| PCV (%)          | 25.6 ± 4.8| 38.6 ± 6.2    | <0.001  | 24.7 ± 3.9  | 37.4 ± 4.1     | <0.001  |
| Ht (cm)          | 130 ± 6.0 | 133 ± 29      | NS      | 133 ± 24.0  | 138 ± 22.8     | NS      |
| Wt (kg)          | 27.8 ± 13.2| 31.4 ± 17.8  | NS      | 30.9 ± 15.6 | 34.5 ± 15.5    | NS      |
| BMI (kg/m²)      | 15.5 ± 1.8 | 16.3 ± 2.9    | NS      | 16.2 ± 3.7  | 17.0 ± 3.3     | NS      |
| FFM (kg)         | 20.5 ± 10.9| 23.8 ± 14.5   | NS      | 20.2 ± 10.6 | 23.6 ± 10.2    | NS      |
| %FFM             | 72.1 ± 6.6 | 74.1 ± 11     | NS      | 65.4 ± 6.4  | 68.8 ± 8.0     | NS      |
| BF (kg)          | 7.3 ± 2.9  | 7.6 ± 4.5     | NS      | 10.6 ± 6.0  | 10.9 ± 5.9     | NS      |
| % BF             | 27.9 ± 6.6 | 25.9 ± 11.4   | NS      | 34.6 ± 6.4  | 31.2 ± 8.0     | NS      |
| PA (deg)         | 5.01 ± 0.97| 5.67 ± 1.23   | 0.02    | 4.62 ± 1.04| 5.79 ± 1.10    | <0.001  |

NS, not significant; PCV, packed cell volume; BMI (body mass index), FFM (fat free mass), BF (body fat), PA (phase angle)
which are derived from α-linolenic acid: eicosapentaenoic acid, 20:5n-3, p < 0.001; docosapentaenoic acid, 22:5n-3, p < 0.001; and docosahexaenoic acid, 22:6n-3, p < 0.001. The relation between phase angle and docosahexaenoic acid is shown in Figure 3. A significant negative correlation between phase angle and red cell cholesterol content (expressed as µg cholesterol / µg lipid phosphorus) was also obtained (Fig. 4, p = 0.003).

Fat-free mass (FFM), the major determinant of phase angle, accounted for 36% of the variation in phase angle in our subjects. Therefore, the statistical analyses were repeated including FFM in the regression model. When phase angle was the dependent variable and FFM, oleic acid, 20:5n-3, 22:5n-3, and DHA and cholesterol were included as the independent variables, only DHA and FFM remained statistically significant (p = 0.004, p < 0.001, respectively).

**Discussion**

The results of the present study confirm our hypothesis, based on analyses of serum phospholipids conducted in two previous studies of children with SCD in Nigeria [8,21], that red cell phospholipids of children with SCD contain significantly lower proportions of n-3 polyunsaturated fatty acids and higher proportions of saturated fatty acids relative to non-SCD controls. These results are in agreement with those of Connor and coworkers [22] who found similar alterations in the fatty acid composition of the red cell phospholipids of U.S. children with SCD. The red cells of the SCD subjects in the present study also contained significantly higher amounts of cholesterol. More importantly, in the present study we found that both the cholesterol content and the percentage of several n-3 fatty acids in the red cells of the subjects were significantly correlated with phase angle. Overall, in the SCD children we studied, cell membrane components that are known to decrease membrane fluidity, such as cholesterol and saturated fatty acids, were associated with a low phase angle while those which increase fluidity, such as long-chain n-3 polyunsaturated fatty acids, were associated with a high phase angle.
The altered fatty acid composition in both the serum and red cell phospholipids of the SCD subjects we studied may be due to several causes. These include: differences in dietary intake between SCD patients and controls, aberrations of the desaturase-elongase pathway that interconverting n-3 and n-6 polyunsaturated fatty acids in SCD, and increased metabolism (e.g., β-oxidation) of the long-chain polyunsaturated fatty acids in SCD. Although we did not obtain dietary information for the subjects in this study, we assumed that the availability of foods containing the essential and long-chain fatty acids did not differ between SCD patients and controls. However, the SCD subjects may have been consuming less food, on average, than the controls.

With regard to the metabolism of fatty acids mentioned above, the activities of the enzymes that are responsible for the elongation and desaturation of the essential fatty acids to produce the long-chain polyunsaturated fatty acids may vary between SCD subjects and controls. In a study of children with protein-energy malnutrition, Decsi and coworkers [23] used product/substrate ratios of fatty acids in serum phospholipids to speculate that children with protein-energy malnutrition have a reduced level of Δ5-desaturase activity compared to well-nourished children. This particular desaturase is a regulatory enzyme in the pathway responsible for the biosynthesis of long-chain polyunsaturated fatty acids in both the n-6 and n-3 fatty acid families [24]. Since children with SCD exhibit some of the characteristics of malnutrition, such as lower weight-for-age and lower lean body mass, the activity of the Δ5-desaturase in these children may be decreased, resulting in lower proportions of the long-chain.
polyunsaturated fatty acids in both serum and membrane phospholipids.

Two important determinants of membrane fluidity are the cholesterol content and the proportions of the fatty acids that comprise the phospholipids of membranes. One way to compare the fluidity of membranes between two or more populations is to determine the mean melting point (MMP) of the fatty acids in the total phospholipid fraction of the membrane. For such a purpose, we used the method of Jensen and Patton [20] to estimate the MMP of red cell phospholipids. We found that the MMP of the membrane phospholipids of the children with SCD was significantly higher compared to controls, indicating a decrease in membrane fluidity in the SCD subjects. A negative correlation was also observed between MMP and phase angle. This observation suggests that the chemical composition and properties of tissue membranes, specifically the acyl chains of the membrane phospholipids, directly influence the magnitude of the phase angle of an individual.

When we examined the relation between phase angle and red cell cholesterol, a significant negative correlation was obtained between phase angle and red cell cholesterol for the total pool of subjects (SCD patients plus controls) \( p = 0.003 \). However, when regression models were used that included cholesterol, DHA, and FFM, only DHA and FFM were found to be significantly correlated with phase angle.

The implications of the observed alterations in the membrane lipids of children with SCD are not known; however, by affecting the physical properties of tissue membranes, including fluidity and capacitance, these changes could compromise various membrane functions. In an in vitro study using red cells from both healthy subjects and patients with SCD, Kuypers and coworkers [25] showed that the deformability and stability of SCD red cells could be altered by changing the fatty acid composition of the phosphatidylcholine (PC) molecules of the membrane. For example, when they replaced the native PC with 1-palmitoyl, 2-arachidonoyl-PC in normal cells, the cells exhibited decreased osmotic fragility without a change in hydration. However, replacement of native PC with 1,2-dipalmitoyl-PC resulted in an increase in both osmotic fragility and cellular hydration. The authors concluded that the state of cellular hydration of sickle cells may be modulated by altering the molecular species composition of the membrane's phospholipids. In a study of subjects with type 2 diabetes, Borkman and coworkers [26] found that decreased insulin sensitivity was associated with a decrease in the proportions of polyunsaturated fatty acids in skeletal-muscle phospholipids. They speculated that changes in the fatty acid composition of muscle membranes could affect insulin sensitivity.

We reported previously significantly lower phase angle values in both African American and Nigerian children with SCD [18,27]. Because phase angle is related to the amount of lean body tissue and the corresponding amounts of tissue membranes [28], it could be that the lower phase angle we observed previously in both the Nigerian and African American children were the result of the lower FFM of SCD children compared to their corresponding controls. Patients with SCD, particularly males over the age of 10 years, usually have significantly less FFM than their healthy counterparts [29]. However, in the present study, although there were no significant differences in the mean weight, BMI, or FFM of the controls and the SCD subjects, nevertheless, we still observed a statistically significant difference in the calculated phase angle of the two groups. We suggest, therefore, that factors other than FFM and which are not related to body size, such as the lipid composition of cell membranes, can influence the phase angle of individuals.

It is reasonable to ask whether dietary modification or fatty acid supplementation might change the fatty acid composition of tissue phospholipids in children with SCD. In two separate supplementation studies involving SCD subjects, changes in the fatty acid composition of red cell phospholipids were obtained after supplementation with...
fish oil containing high amounts of long-chain polyunsaturated n-3 fatty acids. In the first study, Muskeit and coworkers [30] supplemented 13 SCD patients for 7 months with capsules containing eicosapentaenoic acid, docosahexaenoic acid and vitamin E. This supplementation regimen resulted in increased incorporation of n-3 fatty acids in both plasma cholesterol esters and red blood cells. The double-bond index of the red cells increased, indicating an increase in red cell membrane fluidity and deformability. In the second study, Tomer and coworkers [31] also demonstrated changes in platelet and red cell activation markers in subjects with SCD following fish oil supplementation for one year, resulting in fewer pain episodes compared to subjects supplemented with n-6 fatty acids.

In summary, we have shown in children with SCD that the lipid composition of their cellular membranes is related to phase angle, a non-invasive whole body measurement of membrane integrity and function. Membrane function is altered in a variety of clinical conditions and the phase angle may provide a rapid, non-invasive means of monitoring the effect of therapies aimed at improving the health of individuals in whom cell membrane function may be deranged. Although it is known that supplementation with n-3 fatty acids produces changes in the membrane composition of cells, it has yet to be determined whether phase angle can be used as an alternative approach for monitoring these changes. Studies of this nature are in progress.

**Abbreviations**

SCD, sickle cell disease; R, resistance; Xc, reactance; MMP, mean melting point; FM, fat-free mass; BMI, body mass index; DHA, docosahexaenoic acid; PC, phosphatidylcholine.

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