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EFFECT OF NICOTINIC ACID ON HAEMOGLOBIN-S (HbS) GELATION AND OSMOTIC FRAGILITY OF HbS ERYTHROCYTES

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ABSTRACT

Various concentrations (1.00, 1.25, 1.67, 2.50, 5.00 and 10mM) of nicotinic acid were tested for their possible effects on HbS gelation (polymerization) and osmotic fragility rate of HbS erythrocytes. The results indicated an inhibition of HbS gelation by nicotinic acid in a concentration-dependent manner. For instance, at concentrations of 1mM and 10mM nicotinic acid, HbS gelation was reduced by 8.93% and 21.43% respectively, after a 10 minute incubation.

Similarly, a significant ($P<0.05$) reduction in HbS erythrocyte fragility was observed in the presence of the vitamin. The reduction in HbS erythrocyte fragility was also concentration-dependent. For instance, at saline concentration of 0.3% and at nicotinic acid concentrations of 1mM and 10mM, HbS erythrocyte haemolysis was reduced by 18.48% and 50.00% respectively. Nicotinic acid could therefore be considered a necessary and beneficial factor in the diet of sickle cell patients.

Key words: nicotinic acid, haemoglobin S, gelation, osmotic fragility, erythrocytes

INTRODUCTION

Nicotinic acid is pyridine beta-carboxylic acid while nicotinamide is the corresponding amide derivative. Both are white crystalline solids that are freely soluble in water (Anosike, 1994). They are stable when heated or exposed to light, air or alkalis. Nicotinic acid and nicotinamide have equal activity nutritionally since both can be used for the purpose of their coenzyme synthesis. Niacin is frequently used to refer to the two active compounds – nicotinic acid and nicotinamide (Obidoa, 1994). Nicotinic acid as niacin participates directly in hydrogen transfer reactions, a function that is the actual primary step in energy capture reactions in intermediary metabolism during hydrogenation steps (Anosike, 1994).

A deficiency of niacin causes pellagra, a disease condition which affects the skin (discolouration), the digestive system (diarrhea), and the nervous system (dementia). The disease can be cured by feeding tryptophan or by the administration of nicotinamide as a drug to patients (Obidoa, 1994).

Sickle cell haemoglobin (HbS) is a type of abnormal haemoglobin occurring in the erythrocytes of sickle cell disease (SCD) (haemoglobinopathic) subjects, provoking sickling of erythrocytes into elongated crescent shape a change from the normal biconcave disc upon deoxygenation. The end result is polymerization into crystals (Noguchi and Schechter, 1981; Luzzatto, 1981). The resultant sickle erythrocyte deformity produces the cause of all the remarkable crises experienced by SCD subjects (Hebbel *et al*, 1981).

With the understanding of sickle cell haemoglobin as a genetic complication, the management of SCD in the present time is towards general supportive therapy and

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treatment of the sequential disorder (Charache *et al.*, 1984). In recognition of this, experimental studies have been done in recent years exploring certain foods, food and drug additives or constituents (Ekeke and Nsirim, 1987; Ekeke and Ibeh, 1988, Ekeke *et al.*, 2000). The present report is on findings of our investigation of nicotinic acid, a water-soluble vitamin, for its possible effect on sickle cell haemoglobin gelation and the osmotic fragility of HbS erythrocytes, with the possibility that it may be of use in the management of SCD.

MATERIALS AND METHODS

Sodium chloride, sodium metabisulphite, phenylalanine, nicotinic acid and ethylene diamine tetraacetic acid (EDTA) were obtained from Sigma Chemical Company, St Louis Missouri, USA.

BLOOD COLLECTION

The sampled blood from subjects of both sexes and aged between 3 and 26 years were collected into heparin bottles. The blood samples which were confirmed as HbSS blood using standard haemoglobin electrophoresis, were supplied by the Haematology unit of University of Port Harcourt Teaching Hospital, Port Harcourt, Nigeria. All osmotic fragility experiments were carried out with freshly

Table 1a: Effect of Nicotinic acid on Osmotic fragility of HbS erythrocytes (Optical density approach; Mean ±SD; n=10).
Test

% Salinity	Control	10.00mM	5.00mM	2.50mM	1.67mM	1.25mM	1.00mM
1.0%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.9%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.8%		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.7%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.6%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.5%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.4%	0.50 ± 0.01^{a}	0.10 ± 0.01^{f}	0.12 ± 0.00^{f}	0.19 ± 0.01^{e}	0.24 ± 0.02^{d}	0.30 ± 0.03^{c}	0.35 ± 0.02^{b}
0.3%	0.58 ± 0.02^{a}	0.12 ± 0.00^{g}	0.16 ± 0.02^{f}	0.25 ± 0.03^{e}	0.30 ± 0.01^{d}	0.34 ± 0.01^{c}	0.41 ± 0.02^{b}
0.2%	0.64 ± 0.02^{a}	0.15 ± 0.02^{e}	0.18 ± 0.01^{e}	0.32 ± 0.02^{d}	0.35 ± 0.03^{d}	0.40 ± 0.04^{c}	0.47 ± 0.03^{b}
0.1%	0.85 ± 0.03^{a}	0.25 ± 0.01^{g}	0.29 ± 0.02^{f}	0.35 ± 0.01^{e}	0.45 ± 0.03^{d}	0.50 ± 0.02^{c}	0.58 ± 0.01^{b}
0.0%	0.92 ± 0.02^{a}	0.28 ± 0.02^{f}	0.31 ± 0.02^{f}	0.48 ± 0.03^{e}	0.56 ± 0.003^{d}	0.61 ± 0.02^{c}	0.69 ± 0.03^{b}

Note: 0.0% salinity = Deionized water; 1.0% salinity = 10.0g/L Nacl.
Values on the same row having different superscripts are statistically significant at 95% confidence level (P = 0.05)

Table 1b: Effect of Nicotinic acid on percentage fragility of HbS erythrocyte (mean, n=10)

% Salinity	% Rate of Haemolysis (mean, n = 10) at different nicotinic acid concentrations						
	Control	10mM	15mM	2.5mM	1.67mM	1.25mM	1.00mM
1.0%	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.9%	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.8%	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.7%	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.6%	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5%	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.4%	54.35^{a}	10.87^{f}	13.10^{f}	20.65^{e}	26.09^{d}	32.70^{c}	38.04^{d}
0.3%	63.04^{a}	13.04^{g}	17.39^{f}	27.20^{e}	32.61^{d}	36.96^{c}	44.56^{d}
0.2%	77.17^{a}	16.30^{e}	19.57^{e}	34.78^{d}	39.00^{d}	43.47^{c}	51.09^{d}
0.0%	100.00^{a}	30.43^{g}	33.69^{f}	52.18^{e}	60.86^{d}	66.30^{c}	75.00^{d}

Note: % haemolysis = (OD/absorbance of water) x 100; Control = 0.00% nicotinic acid
Values are means of ten different determinations.
Values on the same row having different superscript letters are statistically significant at 95% confidence level (P = 0.05).

Table 2a: Effect of Nicotinic acid on Haemoglobin S polymerization (Mean±SD; n = 10).

Time (Mins)	POLYMERIZATION RATE (OD. APPROACH)							
	Control	Std	Test (Nicotinic acid)					
			10mM	5mM	2.5mM	1.67mM	1.25mM	1.0mM
1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	0.38 ± 0.02^{a}	0.09 ± 0.00^{f}	0.12 ± 0.01^{f}	0.18 ± 0.03^{e}	0.20 ± 0.01^{d,e}	0.23 ± 0.03^{c,d}	0.25 ± 0.03^{b,c}	0.28 ± 0.02^{b}
3	0.48 ± 0.02^{a}	0.18 ± 0.01^{e}	0.26 ± 0.01^{d}	0.31 ± 0.01^{d}	0.33 ± 0.03^{c}	0.37 ± 0.02^{b}	0.40 ± 0.02^{b}	0.45 ± 0.03^{a}
4	0.60 ± 0.01^{a}	0.22 ± 0.02^{e}	0.36 ± 0.01^{d}	0.40 ± 0.02^{c,d}	0.41 ± 0.03^{c}	0.43 ± 0.04^{b,c}	0.44 ± 0.03^{b,c}	0.47 ± 0.01^{b}
5	0.74 ± 0.03^{a}	0.26 ± 0.02^{e}	0.38 ± 0.03^{d}	0.42 ± 0.03^{d}	0.47 ± 0.04^{c}	0.49 ± 0.02^{c}	0.51 ± 0.02^{b,c}	0.54 ± 0.03^{b}
6	0.85 ± 0.01^{a}	0.30 ± 0.01^{e}	0.45 ± 0.02^{d}	0.49 ± 0.03^{c}	0.52 ± 0.03^{c}	0.56 ± 0.01^{d}	0.60 ± 0.01^{c}	0.63 ± 0.03^{b}
7	0.90 ± 0.04^{a}	0.34 ± 0.03^{e}	0.50 ± 0.04^{d}	0.56 ± 0.04^{c}	0.60 ± 0.02^{b,c}	0.65 ± 0.02^{a,b}	0.69 ± 0.03^{b,c}	0.72 ± 0.04^{b}
8	0.99 ± 0.02^{a}	0.41 ± 0.03^{f}	0.55 ± 0.03^{e}	0.60 ± 0.02^{d}	0.63 ± 0.03^{d}	0.70 ± 0.03^{c}	0.73 ± 0.03^{b,c}	0.75 ± 0.02^{b}
9	1.02 ± 0.03^{a}	0.46 ± 0.02^{f}	0.64 ± 0.02^{e}	0.66 ± 0.03^{e}	0.70 ± 0.02^{d}	0.75 ± 0.02^{c}	0.79 ± 0.02^{b}	0.81 ± 0.02^{b}
10	1.12 ± 0.02^{a}	0.66 ± 0.01^{f}	0.88 ± 0.03^{e}	0.92 ± 0.03^{d,e}	0.96 ± 0.01^{c,d}	0.98 ± 0.04^{b,c}	1.00 ± 0.03^{b,c}	1.03 ± 0.03^{b}

Note. Control = normal saline (9.8g/L NaCl); Std = 10mM phenylalanine

Values in the same row having different superscript letters are statistically significant at 95% confidence level (P = 0.05).

Table 2b: Effect of Nicotinic acid (na) on the relative polymerization rate of sickle cell Haemoglobin (Mean; n = 10) after 10mins incubation

[na] mM	% Polymerization rate	% Decrease in polymerization
0.00†	100.00†	0.00†
1.00	91.07*	8.93*
1.25	89.29*	10.71*
1.67	87.50*	12.50*
2.50	85.71*	14.29*
5.00	82.14*	17.86*
10.00	78.57*	21.43*

† Control

* Statistically significant from the control at 95% (P = 0.05) confidence level.

heparinized blood.

For haemoglobin polymerization experiments, the heparinized blood was transferred to a centrifuge tube, and the upper level of the blood was marked on the tube with a felt-tipped marker. After centrifugation for 10 minutes at 1200g, using an MSE Minor centrifuge, the plasma was removed with the aid of Pasteur pipettes. An equivalent volume of isotonic saline (0.98% NaCl) was added to the mark in the centrifuge tube, and the erythrocytes were suspended in the saline solution by repeated inversions of the tube. The erythrocyte suspension was then frozen at 0°C and subsequently thawed out to produce a haemolysate.

HAEMOGLOBIN GELATION EXPERIMENT

The haemoglobin polymerization (gelation) experiment was based on the method described by Noguchi and Schechter (1985). The underlying principle is that haemoglobin S (HbS) undergoes gelation (polymerization) when deprived of oxygen. Sodium metabisulphite was used as a reductant. Exactly 4.8 mls of 2% sodium metabisulphite, 0.1 ml HbS haemolysate and 0.1 ml of normal saline were quickly mixed in a cuvette and the optical density (absorbance) reading taken at 700 nm and at 2-minute intervals for 20 minutes using a Spectronic 20-DR. This served as the control experiment. For the test experiment, 4.8ml of 2% sodium metabisulphite, 0.1 ml of HbS haemolysate and 0.1 ml of the test compound (nicotinic acid) were mixed in a cuvette and the absorbance readings taken as was for the control. Six different concentrations (1.00, 1.25, 2.50, 5.00 and 10.00 mM) of the test compound

(nicotinic acid) served as test samples. As a reference standard, 10mM phenylalanine was used.

OSMOTIC FRAGILITY EXPERIMENT

This was carried out using the method reported by Dacie *et al.*, (1981). For this, exactly 5.0 ml each of ten different saline solutions (1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1%) were delivered into ten different 1x12 cm test tubes. To another tube (#11) was delivered 5.0 ml of distilled water (the #11 tube served as the control). To each of the various test tubes, 50 ul of well-mixed fresh blood was then added.

The whole mixtures were thoroughly but carefully mixed to avoid foaming. After incubation at room temperature for 30 minutes, the mixtures were each mixed again before centrifugation at 1200g for 5 minutes using the MSE Minor centrifuge. Using tube 1 supernatant (osmotic equivalent to 1.0g/l NaC1) as blank, the supernatants of the other tubes were decanted and spectrophotometrically estimated by measuring the optical density readings at 540nm (with a spectronic 20 DR) for the amount of lysis. This served as the control experiment. The test experiments were carried out by repeating the procedure at 0.1ml of different test concentrations (1.00, 1.25, 1.67, 2.50, 5.0 and 10.0mM) of nicotinic acid

STATISTICAL ANALYSIS:

This was done using the students t-test of statistical significance at 95% confidence level (P = 0.05) (Brokes *et al.* 1979). Data were also analyzed by one-way analysis of variance (ANOVA) using SPSS/PC+ package, and differences between means were compared using Duncan's (1955) multiple range test.

RESULTS.

The results of the osmotic fragility experiment are presented in Tables 1a and 1b. The introduction of increasing concentration of nicotinic acid was observed to produce a progressive decrease in red cell lysis. Lysis at 0.3% salinity (0.3g/100ml NaC1) was used as analytical reference for the mean corpuscular fragility. At 0.3% salinity, the control recorded 63.04% fragility of HbSS erythrocytes while percentage fragilities of 44.56, 27.20 and 13.04 were recorded in the presence of 1.00, 2.50 and 10.00 mM nicotinic acid, respectively. Similar results were obtained at other levels of salinity.

Results for the HbS polymerization studies are presented in Tables 2a and 2b. As was the case in the osmotic fragility experiments, the presence of increasing concentration of nicotinic acid recorded a progressive decrease in HbS polymerization (Table 2b). For instance, after 10 minutes incubation and at nicotinic acid concentration of 10.00mM, the polymerization of HbS was reduced by 21.43% relative to the control (0.00mM nicotinic acid). The results are significant at 95% confidence level (P = 0.05).

DISCUSSION

From the results obtained, it was observed that the presence of nicotinic acid at varying concentrations in blood *in vitro* produced reductions in both the HbSS erythrocyte osmotic fragility rate and HbS polymerization rate, and thus by extrapolation, a possible physiological significance of anti-sickling action *in vivo*.

Haemoglobin S (HbS) erythrocytes have been noted to be more prone to lysis (increased fragility) as a result of increased polymerization of HbS with consequent generation of fibrous HbS molecules at states of hypoxia (Diggs, 1939; Uwakwe and Nwinuka, 2000). The finding in this study of the ability of nicotinic acid (a water-soluble vitamin and hence polar compound) to inhibit HbS polymerization and consequently reduce HbSS erythrocyte sickling and eventual fragility is of significance considering the current attention focused on the search for a non-toxic, nutritional prophylactic treatment of sickle cell disease by contemporary scientists (Ekeke, 1999).

Of recent, some nutrient amino acids

(specifically the polar amino acids, phenyl alanine, tryptophan, tyrosine, asparagine and glutamine) have been found to exhibit anti-sickling properties especially in terms of HbS polymerization inhibition as well as HbSS erythrocyte sickling inhibition (Noguchi and Schechter, 1981). More recently, the amino acid glutamate (a polar amino acid) in its form as monosodium glutamate (used as a food seasoning agent) has also been noted to posses remarkable anti-sickling activity (Uwakwe and Nwinuka, 2000). It is therefore not surprising that nicotinic acid, a polar compound and a nutrient vitamin, could posses anti-sickling activity. Although the exact manner in which nicotinic acid exhibits its anti-sickling property was not investigated, it has been suggested that a binding site common to all compounds with anti-sickling activity is at or near the haem pockets in the alpha and beta chains of both deoxy-HbS and deoxy-HbA (Russu *et.al.*, 1986).

It could therefore be possible that the HbS polymerization inhibition as well as HbS erythrocyte stability effect of nicotinic acid is most probably achieved through the binding of the nicotinate ion at or near the haem pockets. This binding could be viewed as constituting a steric hindrance which inhibits HbS gelation (polymerization) and thus result in the maintenance of a stable red cell integrity which was manifested in the reduced lysis (fragility) in the presence of nicotinic acid.

It is thus hoped that this finding when fully established, would be exploited towards successful nutritional management of sickle cell disease, since nicotinic acid (especially as component of multi-vitamin preparations) is a commonly consumed vitamin supplement.

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