

OSMOTIC FRAGILITY AND Na⁺-K⁺ ATPase ACTIVITY OF ERYTHROCYTES OF HIV/AIDS PATIENTS

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A cross sectional study was carried out to investigate the osmotic fragility and Na⁺-K⁺ ATPase activity of the erythrocytes of HIV/AIDS patients. Whole blood was taken from subjects at the Human Virology Laboratory of the Nigerian Institute of Medical Research. Subjects were judged suitable for the various investigations by means of a questionnaire. The Genie II HIV diagnostic kit was used to confirm HIV positive status. HIV positive subjects were grouped into two: those receiving anti-retroviral therapy were referred to as the ARV group and those not receiving antiretroviral therapy were designated as non-ARV group. Each group was further sub-divided according to the Centers for Disease Control 1993 classification of HIV disease. HIV negative subjects must have tested no later than two months to the sample collection date and must not lead a high-risk lifestyle. Twenty microliters of whole blood were used for the erythrocytes osmotic fragility assay. One milliliter of whole blood was used to prepare the erythrocyte ghost membrane for the Na⁺-K⁺ ATPase activity assay. The two HIV positive groups showed significant increase in percentage haemolysis under osmotic stress at 0.65% saline. The ARV group had an average percentage haemolysis of 2.56 ± 0.81% while the non-ARV group had an average of 3.19 ± 1.11% compared to an average of 0.83 ± 0.36% for the control group (p < 0.05). A pattern observed in the result was an increase in activity with increasing severity of the HIV/AIDS disease. Data from the present study indicate that the osmotic fragility of erythrocytes was significantly potentiated, while Na⁺-K⁺ ATPase activity was not significantly altered (p < 0.05) in HIV/AIDS disease.

Key words: Osmotic fragility, Na⁺-K⁺ ATPase activity, erythrocytes, HIV/AIDS

INTRODUCTION

Symptomatology of HIV/AIDS is very diverse. However, anaemia is one of the most universal clinical symptoms of the disease (1). The aetiology of anaemia in HIV disease has been extensively researched primarily from the physiological and pharmacological angles (2,3,4). Malnutrition in HIV/AIDS has also been widely reported (5,6). Biochemically, metallic cofactors and coenzymes obtained through the diet are critical to the proper functioning and integrity of the native conformations of biomolecules. Nutrient deficiencies can result in disruption of supramolecular structures like biomembranes. We therefore decided to investigate the osmotic fragility of erythrocytes of HIV/AIDS patients. However, since the Na⁺-K⁺ ATPase is the major

transmembrane pump involved in regulating osmosis in the cell, we considered it pertinent to determine its activity in the erythrocytes of HIV/AIDS patients.

SUBJECTS AND METHODS

Study design

This was a cross sectional study involving 67 HIV seropositive subjects recruited and confirmed positive at the Nigerian Institute of Medical Research (NIMR), Human Virology laboratory in Lagos, Nigeria. Ten HIV seronegative subjects served as the control group. The subjects were representative of the geo-political, ethnic, economic, religious and education diversity of Nigeria. The purpose of the study was clearly explained to them. Consent was obtained and

counseling given before blood samples were taken. Participants who were on multivitamins or prolonged non-AIDS related treatment were excluded from the study. The age range was between 20 and 60 years. The HIV positive group was sub-divided into those who were on antiretroviral therapy (ARV) at the Institute and those who had not commenced any form of antiretroviral therapy (non-ARV).

The HIV disease-stage classification was according to the Centers for Disease Control revised 1993 classification for HIV infection among adolescents and adults (7). Subjects were deemed unsuitable for the study if they were on mineral supplements and/or cardiac glycosides. Some minerals have been reported to reverse osmotic fragility in erythrocytes and cardiac glycosides are specific inhibitors of Na⁺-K⁺ ATPase activity (8,9).

Blood collection

Blood samples were collected between 08:30h and 09:30h daily. Six milliliters of blood were collected by venous puncture into potassium EDTA bottles. Four ml were aliquoted for CD4⁺ T-lymphocyte count within 6h of collection; 1 ml for erythrocyte ghost membrane preparation while the remaining 1 ml was used for osmotic fragility assay. All tests were conducted on the day of collection. Two ml of blood were taken from the control subject since CD4⁺ counts were not conducted on them.

HIV confirmation

Subjects were screened for HIV status at various centers outside the Institute but confirmation was done at the Virology Laboratory with the Genie II HIV confirmation kit. CD4⁺ counts were performed with the Dynabeads method.

Osmotic fragility assay

Osmotic fragility of the erythrocytes was determined by the method of March *et al* (10). Saline solution of concentrations 0.85%, 0.65%, 0.35%, and 0.10% were prepared. To each dilution series, 20 µL of freshly collected blood was added and all the tubes were shaken gently. They were incubated at 37°C for two hours after which they were centrifuged at 3,000 rpm in a refrigerated desk centrifuge and the absorbance of the supernatant measured at 583nm. The absorbance obtained at 0.85% was subtracted from absorbance at the other concentrations. The result at each concentration was then expressed as a percentage of the highest absorbance. This represents the degree of haemolysis.

Erythrocyte Ghost Membrane (EGM) Preparation

One ml of freshly collected whole blood was used for the EGM preparation. The whole blood was centrifuged at 3,000 rpm for 10 minutes. The plasma was removed to obtain the packed erythrocytes. The erythrocytes were washed twice in five times volume of isotonic buffer at 4,200 rpm for 20 minutes. The supernatant was decanted and the pelleted cells haemolysed in five times volume of hypotonic buffer and centrifuged at 4,200 rpm for 20 minutes. This was repeated four times and the supernatant decanted each time. The pink ghost was then washed five times in four times volume of washing buffer at 4 200 rpm for 20 minutes each. The supernatants were decanted. The entire washing process was done in Jouan CR3i refrigerated centrifuge at 4°C.

Determination of Na⁺ -K⁺ ATPase Activity

Na⁺-K⁺ ATPase activity was determined by a modification of the method of Bowler and Tirri (11). It was calculated as the difference between total ATPase activity and Quabain-inhibited activity. Total ATPase activity was assayed in an incubation medium consisting of 50mM Tris-HCl (pH 7.4), 120mM NaCl, 20mM KCl, 4mM MgCl₂, 240mM sucrose, 1mM EDTA and 3mM disodium ATP. 50 μL of EGM suspension were aliquoted into two tubes labeled 1 and 2. 100 μL of incubation medium were also added to each tube but 100 μL of 1mM Quabain solution was added to tube 2 only. The reaction mixtures were incubated at 37°C for 20 minutes. They were stopped by adding 100 μL of 1% SDS.

Assay for Inorganic Phosphate

Inorganic phosphate produced from the hydrolysis of ATP by ATPase was assayed by the method of Fiske-Subbarow (12). 2.5% ammonium molybdate in 0.9M H₂ SO₄ was added to the EGM reaction mixture after it was stopped. To each tube was added 1 ml of ammonium molybdate and 9% ascorbate. The blue color that developed was read spectrophotometrically at 640nm.

Determination of Protein Concentration

The protein concentration of the EGM was determined according to Lowry *et al* (13), using bovine serum albumin as standard protein.

Statistical Analysis

Data obtained were analyzed by two-tailed Student's t-test (14). A p-value of < 0.05 was considered statistically significant. Calculations were done using Microsoft Excel 2000 statistical tools.

RESULTS

Sixty-seven HIV positive subjects were recruited for this study. During the process of HIV confirmatory tests, 2 subjects were found to be HIV negative and were screened out but one of them joined the control group. Also screened out were 9 subjects who were on cardiac glycosides (6 subjects) and mineral supplements (3 subjects). The remaining HIV positive subjects were grouped either as ARV (35 subjects) or non-ARV (21 subjects). At 0.65% hypotonic saline concentration, the erythrocytes of the non-ARV and ARV groups were found to have higher percentages of haemolysis than the control group (Table 1). Both groups showed statistically significant results (p<0.05).

The Na⁺-K⁺ ATPase activities for the non-ARV and ARV groups were higher than the activity of the control group (Table 2). The difference was however not statistically significant (p<0.05). On detailed investigation, the Na⁺ -K⁺ ATPase of non-ARV and ARV subjects with CD4⁺ T-lymphocyte count of less than 200 cells/μL of blood showed higher activity than subjects with CD4⁺ count of between 200-499 cells/μL (Table 3).

TABLE 1

Degree of Hemolysis of Erythrocytes of HIV-Negative (Control), Non-ARV, and ARV HIV/AIDS Patient in 0.65% Saline Solution¹

Subject	Percent Hemolysis	n
Control	0.83±0.36	10
Non-ARV	3.19±1.11	21
ARV	2.56±0.81	35

¹Value represent mean ± standard error of mean

Control = HIV negative subjects

Non-ARV = HIV positive patients not on antiretroviral therapy

ARV = HIV positive patients on antiretroviral therapy

n = number of subjects, p<0.05

TABLE 2

Na⁺ - K⁺ ATPase Activity of ARV, Non-ARV, and HIV Negative Subjects

Subject	Na ⁺ - K ⁺ ATPase Activity (nmol Pi/h/mg protein)	n
Control	2.22±0.81	10
Non-ARV	3.01±0.52	21
ARV	3.69±0.58	35

¹Value represent mean ± standard error of mean

Control = HIV negative subjects

Non-ARV = HIV positive patients not on antiretroviral therapy

ARV = HIV positive patients on antiretroviral therapy

n = number of subjects, p<0.05

TABLE 3

Na⁺ - K⁺ ATPase Activity of ARV and ARV subjects based on CD4⁺ Count Classification¹

CD4 ⁺ Cell Count (cells/μl)	Non-ARV	n
<200	3.38±0.79	3.99±1.16
200 - 499	2.58±0.71	3.73±0.84
≥ 500	β	γ

¹Value represent mean ± standard error of mean, expressed as nMol Pi/h/mg protein

^a Three of ARV subjects did not have CD4⁺ count done

β There was no subject in this category

γ There was only one subject hence no statistical analysis could be done

DISCUSSION

The erythrocytes of the two groups of HIV positive subjects in this study showed significant susceptibility to osmotic stress. Those who had not commenced any form of antiretroviral treatment (the non-ARV group) had a greater degree of erythrocytes fragility than the group on antiretroviral drugs (the ARV group). Osmotic fragility had been associated with lower concentration of protein sulfhydryls in erythrocyte ghost membranes (8). Xia *et al* suggested in their report that an important function of zinc is to protect cysteine residues in critical plasma membrane proteins from auto-oxidation. Auto-oxidation of the cysteine residues will ultimately lead to a significant conformational change in these proteins, which may in turn cause structural fragility of the plasma membrane.

Micronutrient deficiency has been reported in AIDS patients (5). The deficient micronutrients include zinc, vitamin A, iron, iodine, and trace elements (6). These nutrients are essential as cofactors for the proper functioning and structural integrity of various biomolecules, especially proteins. Some act as antioxidants. Deficiency in a critical micronutrient can completely upset the homeostatic functioning of a cell or the entire organism. This deficiency could be due to reduced intake, increased utilization (15) or urinary excretion (16, 17). The report that reduced micronutrient intake may lead to nutritional deficiency lends credence to osmotic fragility of erythrocytes observed in the HIV positive subjects. Anorexia, nausea, vomiting, and diarrhea are conditions that can result in reduced nutrient intake, which leads to malnutrition. These secondary symptoms

have been observed and reported in acute and late stage HIV disease (1).

It may seem surprising that patients who are undergoing antiretroviral therapy, and are showing significant improvement in health, should also be significantly susceptible to osmotic stress. These patients may have osmotically fragile cells also as a result of micronutrient deficiency. Stephensen *et al* (16) and Jordao *et al* (17) reported that deficiency might occur in HIV/AIDS as a result of increased urinary excretion. Antiretroviral therapy involves a cocktail of drugs taken under a strict regimen. In an attempt to detoxify and/or metabolize these drugs, the liver increases their water solubility (18). Ultimately there is an increase in urine production and a depletory loss of vital water-soluble nutrients like metallic ions may occur. The patients may therefore suffer from conditions like anemia. Zidovudine (AZT) therapy has been reported to be the commonest cause of anemia in HIV-infected persons (1, 4). This supports our findings of possibility of anemia in patients on antiretroviral therapy. Whereas previous reports have attributed this condition to marrow erythroid hypoplasia, aplasia, and megaloblastic maturation (19), we believe that from the present data and cited literatures, osmotic fragility resulting from micronutrient deficiency, is critical to the development of anemia in patients on antiretroviral therapy.

Na⁺ -K⁺ ATPase activities of the erythrocytes were found to be increased in non-ARV and ARV HIV positive patients compared to HIV negative patients, though they were not statistically significant in the present study. The increased activities may be a consequence of the osmotic fragility of the

plasma membrane of the erythrocytes above. The Na⁺-K⁺ ATPase pump is the primary mechanism by which the cell prevents lysis from osmotic stress (20). The activity of the pump increases when the cell is threatened by plasmolysis. The pump performs a continual surveillance role in maintaining normal cell volume. To obtain a detailed insight into these findings, the Na⁺-K⁺ ATPase activities were further analyzed based on the disease stage of the subjects. The criterion used for disease stage classification was the Centers for Disease Control (CDC) revised 1993 classification of HIV disease. The Na⁺-K⁺ ATPase activities increased with the degree of severity of the disease as measured by the CD4⁺ counts. For both the ARV and non-ARV group, the average Na⁺-K⁺ ATPase activity of those with CD4⁺ counts of less than 200 cells/μL of blood was higher than those with CD4⁺ counts of between 200-499 cells. Further, the activity of the ATPase in the two CD4⁺ count classification (200-499 and less than 200 cells/μL) were higher for the untreated HIV/AIDS subjects (non-ARV) than the treated subjects (ARV). Data showed that the Na⁺-K⁺ ATPase activity of HIV/AIDS subjects was slightly elevated with increased severity of the disease. This corroborates the preceding finding that the erythrocytes of HIV/AIDS persons are highly susceptible to osmotic stress and greater so when the disease is left untreated. The plasma membrane becomes highly porous to trans-membrane cationic movement with cations such as Na⁺ and K⁺ moving down their concentration gradients. In an attempt to reverse the resultant hyponatremia of the intracellular fluids, the Na⁺-K⁺ ATPase activity is increased.

In conclusion, data obtained from the present study indicate that osmotic fragility of erythrocytes is significantly increased in HIV disease. The Na⁺-K⁺ ATPase activity of the erythrocytes is only marginally increased in an attempt by the cells to reverse the deleterious effects of osmotic fragility in HIV/AIDS disease.

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