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HAEMORHEOLOGIC AND FIBRINOLYTIC ACTIVITY IN HIV SEROPOSITIVE SUBJECTS AT NNAMDI AZIKIWE UNIVERSITY TEACHING HOSPITAL (NAUTH), NNEWI

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ABSTRACT

Cardiovascular disease has become a major health challenge among HIV patients especially those on antiretroviral therapy (ART). Haemorheology and fibrinolysis are well-defined cardiovascular risk factors. This study was carried out to provide information on the haemorhelogic and fibrinolytic activity in HIV Patients on ART and those not on ART. Blood sample was collected from 160 subjects, 55 HIV-infected patients on Antiretroviral therapy (ART), 55 HIV-infected patients not on ART and 50 apparently healthy HIV Sero-negative individuals as controls. Euglobulin Lysis Time (ELT), Plasma Viscosity (PV), Plasma Fibrinogen Concentration (PFC), CD4 Count, Haematocrit (HCT) and Retroviral screening (RVS) of the subjects were determined using standard methods after obtaining ethics approval and informed consent of the subjects.. ANOVA and t-test were used for statistical analysis. The results showed that ELT, PV, PFC and RDW were significantly higher in HIV infected patients (F=504.74; 130.40; 41.78; 35.63; P<0.05 respectively) compared with control subjects. CD4 was significantly (p<0.05) lower in HIV patients (F=60.57) compared with control subjects.ELT, PV PFC and RDW were significantly higher (P<0.05) in HIV patients on ART (t=10.25; 5.14; 2.11; 5.20) compared with HIV patients not on ART. This study showed that HIV patients especially those on ART have impaired blood flow and derranged fibrinolytic activity which predispose them to increased cardiovascular risk. Hence integration of routine laboratory analysis of haemorheologic and fibrinolytic parameters into HIV management scheme is recommended for more effective HIV patients'care.

KEYWORDS: *Haemorheologic, Fibrinolytic activity, HIV seropositive subjects, Nnewi.*

INTRODUCTION

Human Immunodeficiency Virus (HIV) infection represents the most important infection in the history of mankind (Gustavo, 2012). The high rate of researches on this subject is due to the fact that HIV presents a complex knot for researchers to unravel. The human immunodeficiency virus epidemic has spawned a scientific effort unprecedented in the history of infectious disease research (Coffin, 1999). Despite dramatic advances in basic virology and clinical management, HIV infection has developed into a worldwide pandemic, with tens of millions of individuals infected by the virus and many millions more affected by it. In recent times, it has become one of the world's most serious health challenges and has brought about a global epidemic of massive proportions (UNAIDS, 2014).

HIV is a retrovirus belonging to the family of lentiviruses which are characterized by single stranded RNA genome and long incubation period. HIV causes severe damage to the immune system and if not checked destroys it by using the DNA of the CD4 cells to replicate itself (Dybul *et al*., 2002; Gebo *et al*., 2004).

HIV attacks and destroys the CD4 cells. CD4 cells are T helper cells that lead the attack against infections and form an important component of immune response. Depletion of CD4 lymphocytes is the hall mark of HIV infection and predicts an individual's risk for infection with opportunistic pathogens as well as other complications of HIV infections (Moyle, 2002). The cardiovascular risk of HIV seropositive subjects can be determined by monitoring their haemorheologicl and fibrinolytic activity. Proper tissue perfusion can occur only when blood's rheological properties are within

certain levels. Alterations of these properties play significant roles in disease processes (Monsuez, 2000; Rudnicka *et al*., 2006).

Haemorheology is the study of flow properties of blood and its elements (i.e. blood plasma and cells). It is the study of blood flow in relation to pressure flow, volumes and resistance in blood vessels (Bascurt *et al*., 2007). As long as blood circulates freely and perfuse tissue adequately, the immunological defence system has a good chance of combating infections. However, once the flow becomes sluggish due to increased viscosity, such conditions pave way for diseases like cardiovascular disease (Lenz *et al*., 2008; Von Tempelhiff *et al*., 2000). Blood viscosity is determined by plasma viscosity, hematocrit and mechanical properties of red blood cells. Haemorheological parameters have been associated with cardiovascular diseases (Madjid *et al*., 2004; Tonelli *et al*., 2008). Haemorheological factors are of significance in the determination of flow characteristics of blood and play an important role in the pathogenesis of cerebrovascular diseases (Awodu *et.al*., 2011).

Fibrinolysis is a normal body process where fibrin clot, the product of coagulation is broken down by plasmin at various sites leading to the production of circulating fragments which are cleared by other proteinase or by the kidney and liver (Diez *et al*., 2006; Juhan-Vague *et al*., 1995). Fibrinolytic activity has been postulated as one of the risk factors associated with cardiovascular disease (CVD) (Cesarman and Hajar, 2005). The markers of impaired fibrinolysis and increased CVD risk, the plasminogen activator inhibitor-1(PAI-1) and tissue type plasminogen activator(tPA) antigens are increased in association with hyperinsulinaemia in patients with HIV (Hadigan *et al*., 2001; Palella and Phair, 2011).

AIM

This study is designed to evaluate the haemorheologic and fibrinolytic activity in HIV seropositive subjects attending the out-patients HIV clinic of Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi, Anambra State.

Specific objectives

1. To access haemorheological parameters: - haematocrit (HCT), plasma fibrinogen concentration (PCF), plasma viscosity (PV) of HIV seropositive subjects at NAUTH Nnewi.
2. To measure their fibrinolytic activity using Euglobulin Lysis Time (ELT).
3. To determine the CD4.
4. To compare the haemorheologic and fibrinolytic parameters of HIV seropositive subjects on ART and those not on ART within the study period and check for possible

MATERIALS AND METHODS

Study design

A case-control study design comprising of three groups; HIV seropositive subjects on drugs (ART), those not on drugs (NON ART) and HIV seronegative individuals as control group. The design was to determine the effect of HIV infection as well as ART on haemorheologic and fibrinolytic activities by comparing the effects on the three groups.

Study area

The study was conducted at Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi in Anambra State of Nigeria. NAUTH is a tertiary institution serving patients of high, middle and low socio-economic status. It has about 13,905 enrolled HIV-Infected individuals assessing care at its main branch at Nnewi. The hospital also has about 7,000 HIV-infected individuals enrolled at the five annexes at Ukpo, Umunya, Onitsha, Nnewi and Oba, all in Anambra State.

Ethics consideration

Ethical approval was sought and obtained from the ethical committee of NAUTH Nnewi. The informed consent of the subjects was also obtained for the study.

SAMPLE SIZE CALCULATION

Using the formular $N = Z^2 \times [P(1-P)]/D^2$ (Naing *et al.*, 2008)

N = Minimum sample size

P = Prevalence rate of HIV seropositive subjects = 3.7% (UNAIDS, 2013)

D = Desired level of Significance (5%) = 0.05

Z = Confidence interval (95%) = 1.96

Therefore $N = (1.96)^2 \times [0.037(1-0.037)]/0.05^2$

$= 54.75$

Hence, the minimum sample size was 55

Subject recruitment

One hundred and sixty (160) subjects were recruited and categorized into three groups as follows;

- Fifty-five adult HIV Sero-positive subjects on antiretroviral therapy (ART) Lamivudine (150mg), Zidovudine (400mg), Nevirapine (600mg).
- Fifty-five adult HIV Sero-positive subjects not on antiretroviral therapy.
- Fifty adult Sero-negative control subjects.

Sampling technique

Systematic random sampling method was employed in selecting the participants based on the inclusion criteria.

Sample collection

From each of the subjects, 8ml of blood was collected asceptically by venepuncture. 4.5ml was dispensed into a 0.5ml 3.2% sodium citrate sample container for Euglobulin lysis time (ELT), Relative Plasma Viscosity (RPV) and Plasma Fibrinogen Concentration(PFC) while the remaining 2.5ml was dispensed into EDTA container for CD4 and FBC. Samples for ELT, RPV and PFC were

immediately centrifuged for 25mins at 3000rpm and stored in -20^{0}c freezer while that of CD4 and FBC were analyzed within six hours of collection.

Laboratory analysis

Human Immunodeficiency Virus (HIV) by Serial Algorithm

Serial testing is the WHO standard for HIV testing. It means that when the result of the first test kit shows a non-reactive result, the tested sample was reported as HIV negative; but if the first test kit showed a reactive result, the sample was tested further by a second test kit and if the second test kit showed a reactive result, the tested sample was reported as HIV positive. But when the first two test kits gives a conflicting results (the first is reactive and the second is non-reactive), a third test using a different test kit as the tie-breaker was carried out to give the final result. For this study, Determine, Unigold and Stat-pak HIV ½ test kits were the first, second and tie-breaker respectively.

DETERMINE HIV 1/2 ASSAY

Test procedure

The protective foil was removed and 50µl of the serum sample was applied to the sample pad. The result was read after 15 minutes.

Unigold HIV 1/2 rapid test

Procedure

The test device was removed from the protective wrapper. Over the sample port 60µl of serum sample was added. Also two drops of wash buffer reagent. Result was read after 10 minutes.

Chembio stat-pak HIV 1/2 assay

Test procedure

The test kit was removed from its pouch and placed on a flat surface. The test device was labelled with sample identification number. The 5µl sample loop was touched on the specimen allowing the opening of the loop to be filled. The sample loop was then held vertically to touch the centre of the SAMPLE (S) well of the device to dispense approximately 5µl of sample onto the sample pad. The Running Buffer bottle was held vertically and three drops (approximately 105µl) of the Buffer was added slowly. The test result was read after 10 minutes of adding the Running Buffer.

Determination of Euglobulin Lysis Time by Euglobulin Fraction Method

Test Procedure

Five hundred microliter of citrated plasma was added to 9.5 ml of 1% acetic acid in a test tube, and kept at 4˚c for 30mins to precipitate the euglobulin fraction. The tube was then centrifuged at 2000 rpm for 10mins. The supernatant was discarded and the tube inverted to remove all acetic acid. The deposit was re-constituted with 0.5ml borate buffer. The tube was pre-warmed at 37˚c alongside calcium chloride (0.025M) for 2mins, then 0.5ml of the calcium chloride was added to the tube containing the deposit and borate buffer, mixed and observed for clot. Immediately clot was observed, stop watch was used to measure the time taken for the euglobulin fraction to lyse completely and then recorded.

Determination of Relative plasma viscosity by Simple Viscometer Technique

Test procedure

One milliliter syringe with a hypodermic needle was used to draw plasma into the syringe upto 1ml mark avoiding air bubbles. The plunger was carefully removed and the time taken for the entire plasma to drain was noted. This was done twice for each sample and the average was taken. Then the whole process was repeated using distilled water. The ratio of the flow rate of plasma to that of the water was obtained as the plasma viscosity.

Determination of Plasma Fibrinogen Concentration (Clauss Method)

Procedure

Fifty microlitre of the sample was diluted with 450µl of imidazole buffer (1 in 10 dilution). 200µl of the diluted sample was added to the test cuvettes, the sample was incubated for 5mins at 37oC. Then 100µl of Bovine Thrombin was added and the result of the fibrinogen concentration was automatically calculated by the equipment. The test was performed in duplicate and the average taken.

CD4 Cell Count

This was carried out using the Partec new model Cyflow Counter 2 which is an automated flow cytometer for the enumeration of CD4, CD4 percentage and CD8- T lymphocyte cells in whole blood.

Procedure

Twenty microliter of CD4 PE antibody was added into a partec test tube (rohren tube), then 20ml of well mixed whole blood (EDTA) was also added into the tube. The mixture was gently mixed and incubated in the dark for 15mins at room temperature.

After incubation, 800ml of CD4 buffer was added and mixed gently avoiding air bubbles. The tube was then plugged on to the counter and the start button was clicked to run. After counting, the lymphocytes was well gated and the CD4 value was taken. (Ezenwelu, 2007; Partec Cyflow, 2006).

Statistical analysis

The data obtained was analyzed using Statistical Package for Social Sciences (SPSS) version 20). Data were expressed as mean ± SD. The significance of differences in mean values between groups were analyzed using t-test, while significance of the differences in mean values among different groups was evaluated using one-way ANOVA. $p < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows comparisons of Mean ± SD of ELT,(mins), Relative Plasma Viscosity (RPV), Plasma Fibrinogen Concentration PFC(mg/dl),CD4(cells/µl) and HCT (%) in HIV seropositive subjects on ART (group X), those not on ART (group Y) and Control subjects (HIV seronegative) (group Z).

The mean value of ELT was significantly higher in both group X [483.98 ± 68.13(mins)] and group Y [368.40±49.21(mins)] when compared with the corresponding values in the control [177 ±7.49(mins)]. Similarly, the mean value of Relative Plasma Viscosity were significantly higher in group X (2.57± 0.44) and group Y (2.18 ± 0.34) compared with the control (1.52 ± 0.11). Also, the mean ± SD of Plasma Fibrinogen was significantly higher in group X [4.28 ±1.02(g/l)] and group Y [3.89 ± 0.76(g/l)] compared with the control [2.91 ± 0.41(g/l)]. Moreover, the mean value of the ELT, PV and Fibrinogen were significantly higher in group X compared with group Y ($P < 0.05$). The mean ± SD of CD4 was significantly higher in control [1017.76 ± 256.80(cells/µl)] when compared with the corresponding values in both group X[529.98±299.72(cells/µl)] and Y [495.89±280.54(cells/µl)]. Table 4.1.

TABLE 1: MEAN ± SD OF ELT, RPV, PFC, CD4 AND HCT COMPARED AMONG HIV SEROPOSITIVE SUBJECTS ON ART, NOT ON ART AND HIV SERONEGATIVE SUBJECTS

GROUPS	ELT (mins)	RPV	PFC (g/l)	CD4 (cells/µl)	HCT (l/l)
(X) HIV POS on ART (n=55)	483.98± 68.13[a,b]	2.57 ± 0 .44[a,b]	4.28± 1.02[a,b]	529.98± 299.72[b]	0.37 ± 0.04[a]
(Y)HIV POS not on ART (n=55)	368.40± 49.21[b]	2.18± 0.34[b]	3.89± 0.76[b]	495.89± 280.54[b]	0.34 ± 0.05[b]
(Z)CONTROL (n=50)	177 ± 7.49	1.52 ± 0 .11	2.91± 0.41	1017.76± 256.80	0.38 ± 0.04
F-Values	509.74	130.42	41.78	60.57	8.41
P- Values	0.00*	0.00*	0.00*	0.00*	0.00*

Key:
a = <0.05 when compared with NON ART
b = <0.05 when compared with CONTROL
ELT= Euglobulin Lysis Time
RPV=Relative Plasma Viscosity
PFC=Plasma Fibrinogen Concentration
HCT= Haematocrit

DISCUSSION

Altered haemorheological and derranged fibrinolytic activity have been proven to contribute to cardiovascular diseases. Increase in inflammatory proteins and immunoglobulins produced against HIV or any other opportunistic infection associated with HIV cause defective blood flow (Athanassiou *et al*., 2010).

In this study, factors which directly affect blood flow in vivo like Relative Plasma Viscosity, Fibrinogen concentration, Euglobulin lysis time were found to be abnormal.

Relative Plasma Viscosity was observed to be significantly higher in HIV positive patients than the controls. This is in conformity with earlier reports of Moyle (2002). In HIV infection, there is increase in inflammatory proteins such as immunoglobulin, haptoglobulin and C-reactive proteins which are produced against the HIV virus or any other opportunistic infection associated with HIV. These proteins can cause an increase in plasma viscosity. Plasma viscosity is a useful indicator of acute inflammation which is observed in HIV infection (Omoregie *et al*., 2008). This increase may be a step during atherosclerosis pathogenesis (Abu-Samak *et al*., 2011). Moreover, in this study the plasma viscosity of HIV positive patients on ART were higher compared to those not on ART. This agrees with earlier study done by Jeremiah *et al.* (2012). The work showed that antiretroviral drugs especially protease inhibitors have been implicated in cardiovascular disease.

The Plasma Fibrinogen Concentration was observed to be higher in HIV subjects than the control. It was also higher in HIV seropositive subjects on ART than those not on ART This finding is in consonance with that of Omoregie *et al*. (2008). Fibrinogen is an acute-phase reactant which increases greatly in inflammatory and degenerative conditions such as HIV infection. The increase in plasma viscosity observed among HIV seropositive subjects in this study corresponds with the

observed increase in plasma fibrinogen concentration since fibrinogen concentration significantly affects plasma protein and then plasma viscosity.

Haematocrit in this study was found to be significantly lower in HIV seropositive subjects compared with the controls. This is in agreement with a work done by May *et.al*. (2007). The haematocrit value has effect on the rate of sedimentation of erythrocytes and that affects erythrocyte aggregation leading to impaired blood flow. Also, the haematocrit value for those on ART was significantly higher compared to those not on ART which is in tune with the report of Amegor *et al*. (2009). This is evident that ART has the ability to promote blood cell production (Allen *et al*., 2000).

Any derangement in fibrinolysis can result in thrombosis and cardiovascular problems. In this study, fibrinolytic activity was measured by determining the Euglobulin Lysis Time (ELT). The ELT measured was found to be significantly higher in HIV seropositive subjects than the controls. This hypofibrinolytic state observed in this study is in line with the study done by May *et al*. (2007). This could be due to low level of free tPA observed in HIV seropositive subjects. The HIV seropositive subjects on ART were found to show more pronounced hypofibrinolytic state (higher ELT values) than those not on ART. This could be due to the fact that ART promotes the release of plasminogen activator inhibitor which suppresses the action tissue plasminogen activator leading to reduced fibrinolytic activity(Jeremiah *et al*., 2012). This increase in PAI-1 is a marker of atherothrombotic risk. This finding is also supported by earlier report by Folsom *et al*. (2000).

CD4 count appeared reduced in HIV seropositive subjects compared with the controls. This supports the well documented fact that HIV infection attacks and destroys the CD4 cells which are their primary targets (Daniel *et al*., 2011). In this study, there was no significant difference between the CD4 count of HIV seropositive patients on ART and those not on ART. This could be due to the fact that only patients who present with CD4 < 350 cells/µl are placed on ART meaning that most of the patients not on ART have high CD4 values.

CONCLUSION

This study reveals that HIV seropositive subjects have both abnormal blood flow and impaired fibrinolysis. The patients on ART were found to be at higher than those not on ART and consequently higher cardiovascular risk. Also, duration of ART was found to be positively correlated with ELT. These findings suggests defective blood flow and fibrinolytic activity among HIV seropositive subjects which could dispose them to risk for cardiovascular disease.

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