www.jmscr.igmpublication.org
Impact Factor (SJIF): 6.379
Index Copernicus Value: 79.54
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossrefDOI: https://dx.doi.org/10.18535/jmscr/v6i10.27

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

Antioxidant Profile of HIV Positive Subjects on HAART Attending ART Clinic in Asaba, Delta State

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Abstract
Highly Active Antiretroviral Therapy (HAART) has been found to be very effective in reduction of viral load and progression of HIV disease. It is however believed that this is at the expense of elevated reactive oxygen species (ROS) levels. There are however, not enough data to support this in Nigeria. The aim of this study was to determine the possible effects of Highly Active Antiretroviral Therapy (HAART) on the antioxidant profile of HIV positive patients who attended antiretroviral clinic in Asaba, Delta State, Nigeria. This study examined the modification in the antioxidant profile of HIV positive subjects on HAART treatment in Asaba as compared to the HIV positive subject HAART-naïve. One hundred (100) HIV positive patients on HAART and forty (40) HIV positive HAART-naïve patients who attended the Antiretroviral Therapy clinic in Asaba were studied. Antioxidant profile (Superoxide dismutase activity, Glutathione peroxidase activity) & electrolytes (Calcium, Phosphorus and Magnesium) were determined. The results showed a significant higher values ($p<0.05$) for the HAART treated subjects as against the HAART-naïve participants for SOD(HAART treated:18.65 ± 4.91U/ml vs. HAART-naïve:15.59 ± 3.59U/ml; $p<0.0021$), GPX(HAART treated:1.19 ± 0.69U/ml vs. HAART-naïve:0.77 ± 0.44U/ml; $p<0.001$), Calcium(HAART treated:2.59±0.53mm0l/L vs. HAART-naïve:1.97± 0.25mmom/L; $p<0.0001$), Magnesium(HAART treated:0.97 ± 0.23mmol/L vs. HAART-naïve:0.78 ± 0.17mmol/L; $p<0.0001$), while a significant decrease was observed for Phosphorous(HAART treated:0.92 ± 0.25mmol/L vs. HAART-naïve:1.15 ± 0.46mmol/L; $p<0.039$). These increase in SOD & GPX activities indicates that a substantial oxidative stress occurs in the patients that are being treated with HAARTs in the ART Clinic in Asaba while showing a positive effect on the Calcium, Phosphorus and Magnesium. Conclusively, this study contributes to evidences that HAART further increases the oxidative stress on the HIV positive patients on HAART and the data offers a guide to clinicians and other professionals on the care and management of HIV positive patients that are on HAART treatment in Asaba and by extension in Nigeria.

Keywords: *Antioxidants, HIV, HAART, ART Clinic, Asaba.*

Introduction

WHO policy documents have identified priorities for handling HIV/AIDS aimed at pursuing the goal of "universal access, to comprehensive prevention programs, treatment, care and support" by the year 2010[1] Current data indicates that about 70% of the

burden of HIV is in Sub-Saharan Africa with Nigeria contributing about 9%, and having a national prevalence of 4.1% by 2010[2] Human Immune Deficiency Virus (HIV) neurotoxic proteins are the coat protein, glycoprotein (gp) 120 and the transcription regulator, (Tat). These proteins are responsible for inducing HIV dementia or encephalitis in acquired immune deficiency syndrome (AIDS) patients. Each of this protein can induce apoptosis of cultured neurons and renders it vulnerably excitetotoxic with oxidative stress [3].Regulated Ca^{2+}release from the endoplasmic reticulum(ER) controls many neuronal functions, from plasmalemmal excitability to synaptic plasticity. Enzymatic cascades that are localized in the ER, dependent on the Ca^{2+} concentration in the ER lumen, integrate rapid Ca^{2+}signalling with long-lasting adaptive responses through modifications in protein synthesis and processing. Interactions between $Ca^{2+}$and reactive oxygen species signalling coordinate signalling, which can be either beneficial or detrimental[4].

Results have indicated significantly lower levels of ionic calcium, potassium, magnesium and Sodium in all analyzed biological samples (blood, serum and scalp hair) of male patients with Acquired Immune Deficiency Syndrome (AIDS) in comparison to healthy controls. Electrolyte deficiency has been associated with an increased risk of human.

Mineral homeostasis requires the transport of calcium, magnesium and phosphate across their target cells in bone, intestine and kidney: this transport can also be trans-cellular and around cells (pericellular). The pericellular transport is usually diffusional, down a gradient ("downhill"), and not hormonally regulated.Human immunodeficiency virus (HIV) infection induces a wide array of immunologic alterations among the many already identified mechanisms and cofactors that contribute to the progression of the disease.It was recently suggested that oxidative stress induced by reactive oxygen species (ROS) could play a part in the stimulation of viral replication and in the development of immunodeficiency. Excessive production of ROS (superoxide anion, hydroxyl radical, and hydrogen peroxide) might be explained by a polymorphonuclear leukocyte activation in infectious conditions and/or by a pro-oxidant effect of tumour necrosis factor α (TNF- α) produced by activation macrophages. These ROS can attack bases in nucleic acids, amino acid side chains in proteins and the double bonds in polyunsaturated fatty acids, thereby compromising cell integrity and function. Moreover, ROS can stimulate HIV replication through the activation of the nuclear transcription factor-kB (NF-kB) cell gene as demonstrated in a human cell line [5].However, cells are protected against oxidative damage by defence systems. The first of these involve enzymes that directly metabolize ROS [superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). Among these enzymes, SOD plays a central role in the metabolism of ROS by directly dismuting the superoxide anion radical in hydrogen peroxide, which is scavenged by catalase and GPX. The latter enzyme requires the presence of reduced glutathione (GSH) to be effective. The other defence system includes molecules that interact directly with free radical to neutralize them (eg, ascorbic acid, α-tocopherol, retinol, and glutathione)[6].

Previous studies have shown that HIV-infected patients have deficiencies in their antioxidant systems that could enhance susceptibility to oxidative stress. In particular, decrease concentrations of glutathione[7], selenium, which is essential for GPX activity[8]. A majority of research has focused on the use of highly active antiretroviral therapy (HAART) to suppress HIV viral replication and the progression of HIV disease. The of the current study was to determine the possible effects of Highly ActiveAntiretroviral Therapy (HAART) on the antioxidant profile of HIV patients and correlates the antioxidant with Calcium, Phosphorus & Magnesium.

Materials and Methods

Study Area

The study was carried out in Asaba, Delta state, Nigeria. Asaba, the capital city of Delta State, Nigeria is situated within geographical co-ordinates

6°11′52.23″N6°43′42.48″E. It is situated on a terrace of the lower Niger River, overlooking the point where the Anambra River flows into it. Beyond the river banks, on the high plains which are far more extensive than the river basins, secondary forest vegetation flourishes [9]. The historic Niger River is a trans-African link beginning from West Africa and down into the Atlantic Ocean. Asaba forms a connector between western, eastern and northern Nigeria through the Niger River from the north and via the Asaba Niger Bridge, an east-west link and a Nigerian landmark. The analysis was carried out at Federal Medical Centre, Asaba in Delta State, Nigeria. Federal Medical Centre, Asaba. Established in 1998 as a result of the policy of the Federal Government of Nigeria to establish a Federal Medical Centre in any state where there were no Federal Teaching hospitals.

Ethical Issues

Ethical approval and permission was sort and obtained from the ethical committee of Federal Medical Centre, Asaba. Informed consent of the participants involved was also obtained.

Study Design

A Case-Control observational study design was adopted to effectively execute this study. The variables obtained from positive HIV Subjects on HAART (Case) were compared to positive HIV Subjects who were not on HAART (Control) to establish the relationship.The sample size was calculated using Cochran's sample size formula 20% of the target population to be sampled, with the number of patients on HAART put at about 1,600.By adding 10% of non-respondent, the sample size was calculated to be 130.

Blood Sample Collection

Purposive sampling and randomized method was used in the selection of subject.

10ml of blood was collected from the participants after going through and signing the consent form in a plain container, labelled and allowed to retract. They were then centrifuge for five minutes at 3000rpm. The serum was separated into two plain tube for each patient (one for Calcium, Phoshorus & Magnesium and one for SOD & GPX), labelled and stored at < -15ºC. Calcium, Phoshorus and Magnesium was analysed at Federal Medical Centre, Asaba while sample for SOD & GPX was transported in a cold box to Biotechnology & Research Centre, Nnamdi Azikiwe University, Awka were the analysis was done. Each batch of samples was analysed within one week of collection.

Experimental Analysis

Serum Superoxide Dismutase (SOD):

SOD activity was performed using UV 752 Spectrophotometer (PEC Medical, USA) following the method of [10].

Serum Glutathione Peroxidase (GPX)

Glutathione peroxidase activity was performed using UV 752 Spectrophotometer (PEC Medical, USA) following the method of[11].

Serum Calcium

Calcium was performed by O-Cresolphthalein complexone method using Mindray BS200 Chemistry Auto Analyser and reagent kit from Randox UK. [12].

Serum Phosphorus

Serum Phosphorus was assayed by phosphomolybdate/UV method using Mindray BS200 Chemistry Auto analyser and kitfrom Randox UK.

Serum Magnesium

Magnesium was assayed by Xylidyy Blue method using Mindray BS200 Chemistry Auto analyser and kit from Cypress Diagnostics Belgium.

Serum Albumin

Albumin was assayed by Bromocresol Green method using Mindray BS200 Chemistry Auto analyser and kit from Cypress Diagnostics Belgium.

Statistical Analysis

Data were analysed with Excel 2003 program (Excel Inc.) and Graphpad Prism 6.1, and expressed as mean±SD. Analysis of variance (ANOVA), Student independent t-test & Correlation were used to compare values of measured parameters between HIV positivesubjects on HAART and control subjects (HAART-naïve). P-values less than 0.05 were considered significant. The confidence level was 95%.

Results

The information about the subjects investigated are shown in Tables 1.1, 1.2 & 1.3. Among the 140 HIV positive subjects investigated, 100 are on HAART and 40 are HAART naïve as controls. The mean age in the HAART treated and HAART naïve was 40.41±11.96 years and 38.2±9.04 years respectively. The Antioxidant profile of the two groups are shown in Table 1.1 with those on HAART having a significantly higher values in Calcium (2.59±0.53mmol/l), Magnesium (0.97 ± 0.23mmol/l), SOD (18.65 ± 4.91U/ml) & GPX (1.19 ± 0.69U/ml) as against the HAART-naïve Calcium (1.97 ± 0.25mmol/l), Magnesium(0.78 ± 0.17mmol/l), SOD(15.59 ± 3.59U/ml), GPX(0.77 ± 0.44U/ml). While, a significantly lower phosphorus level (0.92 ± 0.25mmol/l), was observed for the HAART treated as against the HAART-naïve (1.15 ± 0.46mmol/l).

Table 1.1: Comparison between HIV Subjects on HAART and Naive HIV Subjects

Variables	Age(Yr)	Ca^{2+}(mmol/l)	Mg^{2+}(mmol/l)	PO_4^{2-}(mmol/l)	SOD(U/ml)	GPX(U/ml)
HIV Patients on HAART N=100	40.41±11.96	2.59±0.53	0.97±0.23	0.92±0.25	18.65±4.91	1.19±0.69
Naïve-HIV Patients N=40	38.2±9.04	1.97±0.25	0.78 ± 0.17	1.15 ± 0.46	15.59 ± 3.59	0.77±0.44
p-value	0.342	**0.0001***	**0.0001***	**0.039***	**0.0021***	**0.001***

* indicate significant values

Table 1.2 compared the Female and Male HIV subjects on HAART and showed no difference in both group for Calcium, Magnesium, Phosphorus and GPX, however, female is shown to have a slightly higher SOD activity. There was no difference on the profile base on duration of treatment as shown in Table 1.3

Table 1.2: Comparison between Male and Female HIV Subjects on HAART

	Age(Yr)	**Ca^{2+}(mmol/l)**	**Mg^{2+}(mmol/l)**	**PO_4^{2-}(mmol/l)**	**SOD(U/ml)**	**GPX(U/ml)**
Female N=71	38.91±9.87	2.58 ± 0.52	0.96 ± 0.26	0.92 ± 0.22	19.09 ± 5.12	1.17 ± 0.72
Male N= 29	43.54±15.12	2.61 ± 0.56	0.99 ± 0.19	0.93 ± 0.35	17.74 ± 4.38	1.23 ± 0.63
p-value	0.08745	0.804	0.486	0.9322	0.1372	0.6743

Table 1.3: Effect of Duration of HAART on the Stress Profile of Subjects

	Age(Yr)	Ca^{2+}(mmol/l)	Mg^{2+}(mmol/l)	PO_4^{2-}(mmol/l)	SOD(U/ml)	GPX(U/ml)
0-4yrs N= 50	39.83±11.61	2.57 ± 0.57	0.97 ± 0.97	0.93 ± 0.29	18.74 ± 5.32	1.20 ± 0.61
5-9yrsN=35	40.67±11.24	2.6 ± 0.50	0.96 ± 0.27	0.89 ± 0.22	17.99 ± 4.72	1.11 ± 0.72
10 & above N=15	42±15.76	2.67 ± 0.46	1.02 ± 0.19	1.01 ± 0.24	20.31 ± 3.43	1.41 ± 0.86
p-value	0.811	0.774	0.715	0.328	0.28	0.357
F-value	0.209	0.255	0.335	1.122	1.28	1.038

Table 1.4 shows the correlation amongst the subjects on HAART with only Magnesium and Phosphorus showing a significant positive correlation, ($p<0.05$). From the results, SOD shows a positive correlation to GPX, calcium correlate negatively to SOD while magnesium correlate negatively to GPX.

Table 1.4: Correlation among Subjects on HAART

	r	**p-values**
Mg^{2+} & PO_4^{2}	0.196	**0.032***

*indicate significant values

Discussion

The results of this study showed a significant ($p<0.05$) difference in values obtained from the subjects on HAART treatments as compared to the Control group, HAART-naïve subjects as shown in Table 4.1. This study found out that there was a significant higher SOD and GPX activities with subjects on HAART treatments (SOD: 18.65 ± 4.91U/ml), GPX: 1.19 ± 0.69U/ml) as compared to the HAART-naïve (SOD: 15.59 ± 3.59U/ml), GPX: 0.77 ± 0.44U/ml) subjects in Asaba. This suggest that an increased generation of free radicals and a disturbed glutathione metabolism may have occurred after initiation of HAART. This is in line with findings by [13][14]stating that HIV infection can increase the oxidative stress process, which is then further increased by HAART usage. The use of HAART has been associated with some serious side effects which includes increased free radical generation and its attendant free radical injury via Reactive Oxygen Species (ROS)[15]. The use of HAART has also been associated with disturbances in membrane lipid peroxidation and its accompanied alteration in the body's ability to maintain oxidative balance moreover, alterations in some key trace metals with powerful antioxidative property have been reported. Poor nutrition also exacerbate the situation. An *in vitro* study by[16] investigated whether HAART drug combination of AZT and Indinavir (IDV) may alter the blood-brain barrier (BBB) endothelial cells, which may exacerbate this condition. Following 72 hours of treatment, the viability of the cells was significantly reduced in a dose-dependent manner and levels of ROS were highly elevated. AZT+IDV treatment also induced apoptosis in endothelial cells. An additional study investigated the related side effects of Efavirenz (EFV), which is another widely used treatment for HIV-1 infection[17]. Similar to previously discussed study, viability was reduced in a concentration-dependent manner and EFV triggered apoptosis. EFV also lowered cellular proliferation and directly affected mitochondrial function in a reversible fashion by decreasing mitochondrial membrane potential and increasing Superoxide production. As previously demonstrated,[17] found the toxic effect of EFV treatment to be partially reversed by antioxidant pre-treatment. The elevated levels of ROS in each study indicate HAART generates ROS, thereby provoking the onset of OS, which has already been well established to occur upon HIV infection. Hence, while the oxygen faces a paradox, so does HAART: although viral loads may be suppressed, it is at the expense of elevated ROS levels that are known to only activate HIV transcription pathways and promote cell death.

GPX activity was also significantly higher in the HAART-treated than those of the HAART- naïve patients just like SOD.SOD is believed to play a major role in the metabolism of ROS. It is the first enzyme involved in the destruction of superoxide anion radicals by converting it to hydrogen peroxide (H_2O_2), which is then metabolized by catalase and glutathione peroxidase (GPX) in synergy with glutathione (GSH)[6]. The increase in GPX activity value is expected following the increase in SOD activity which mean more hydrogen peroxide are being produced. These effects appears to be related to persistent tumour necrosis factor-α (TNF-α) activation in HIV- infected patients and an up regulate inflammatory cytokine activities in patients receiving HAART[17][18]. N-acetyl-cysteine (NAC) an acetylated precursor of both L-cysteine and reduced glutathione (GSH), maintains intracellular thiol levels during OS, while restoring depleted GSH levels from infection. GSH is one of the body's most important and powerful natural antioxidant and detoxifier. It is known to aid in the transport of nutrients to lymphocytes and phagocytes, as well as in the protection of cell membrane. Oral NAC supplementation has shown positive results in improving the quality of life and well-being of patients faced with a wide range of genetic defects, metabolic disorders, and infections, including HIV infection [19][20].

The findings in this study showed the mean values for Calcium (Ca^{2+}: 1.97 ± 0.25mmol/L), Phosphorus (PO_4^{2-}: 1.15 ± 0.46mmol/L) and (Mg^{2+}: 0.78 ± 0.17mmol/L) obtained for the HIV positive subjects who are HAART naïve were lower when compared

with established reference ranges[(Ca^{2+}: 2.02-2.26mmo/L),(PO_4^{2-}: 0.81-1.45mmol/L) & (Mg^{2+}: 0.66-1.03mmol/L)]. A study by[21] indicated lower calcium, potassium, magnesium amongst male HIV patients and [22] also found lower calcium values in HIV infected individuals. Another study in Nigeria by [23] indicated a lower calcium & phosphorus levels in HIV/AIDS positive patients as compared to healthy HIV negative individuals. However, the study also showed that there was a significant ($p<0.05$) modification of the values for calcium, phosphorus and magnesium[(Ca^{2+}: 2.59 ± 0.53mmol/L), (PO_4^{2-}: 0.92 ± 0.25mmol/L) & (Mg^{2+}: 0.97 ± 0.23mmol/L)] values amongst the HIV positive patient on HAART who attends the ART clinic Asaba as compared to the HIV positive patients who are not yet on HAART. Whereas the Calcium and Magnesium levels increased with the HAART-treated, Phosphorus levels showed a decrease and this is consistent with the normal relationship between Calcium and Phosphorus. Calcium and phosphorous occurs as a compound they are important in bone metabolism, they go hand in hand if one goes up the other goes down and vice versa. Calcium phosphate {Ca^3 $(Po4)^2$} is the principal organic constituent of bones and of bone ash. Phosphorous is more widely distributed than calcium and also serves as a variety of biological functions[24][25]. Calcium and phosphorous, and magnesium are transported to blood from bone, renal and GI cells, and vice versa [26].These transport mechanism can be trans-cellular and around para-cellular. The cellular transport is mediated by the membrane structure and by binding transport proteins[27]. This seem to suggest that the ART drugs may have reversed the distortion in the homeostasis of these electrolytes by reducing viral multiplication. It had also been demonstrated that the viral proteins caused neuronal dysfunction and death in rodents in vivo, and disrupt neuronal calcium homeostasis by perturbing calcium regulating systems in the plasma membrane and endoplasmic reticulum. Other reason could be due to improvement in plasma albumin level which usually closely related to calcium as it has been proved that hypoalbuminaemia in AIDS is caused by recurrent catabolic states, malabsorption, hyper catabolism and decreased oral intake, with clinical appearance of the wasting syndrome[28][29]. The regular counselling, supplements often recommended at the clinic and a change in their dietary habits may also be responsible for the improvement as vitamin D deficiency was found to be a major cause of hypocalcaemia in HIV/AIDS patients [29][30]. Whatever happens to Calcium metabolism invariably affects Inorganic Phosphate in a reverse direction.

Moderate or severe magnesium deficiency is usually due to losses of magnesium from the gastrointestinal (GI) tract or kidneys. Magnesium deficiency is commonly associated with losses from the lower intestine in diarrhoea. Diarrhoea, due to opportunistic infection are common occurrences with HIV positive HAART-naïve patients, because as the viral load increases, the body immunity decreases. Reports have revealed HAART regimens have successfully controlled AIDS and its related disorders, as well as reduced the amount of active virus to undetectable levels at times. This can therefore account for the significant improvement in Magnesium levels. Also, Magnesium metabolism is closely linked to that of calcium. This is because magnesium competitively inhibits the entry of calcium into neurons preventing neuromuscular excitability.

There was no significant ($p<0.05$) correlations between the electrolytes (calcium, phosphorus & magnesium) and the antioxidant enzymes (SOD & GPX) amongst HIV positive patients on HAART, as can be seen in table 1.4. Calcium showed a negative correlation($r = -0.069$, $p<0.455$) with SOD, but a positive one with GPX($r = 0.066$, $p<0.478$), while Magnesium showed a positive correlation with SOD($r = 0.001$, $p<0.988$), but a negative one with GPX($r = -0.118$, $p<0.199$). Phosphorus on the other hand showed a positive correlation with both SOD($r = 0.040$, $p<0.664$) and GPX($r = 0.113$, $p<0.219$). The study however, found a significant correlation between magnesium and phosphorus ($r = 0.16$, $p<0.032$).

The result as shown in table 1.3 found no significant ($p<0.05$) effect on the values as a result of duration of treatment. The values for calcium, magnesium and phosphorus remained virtually unchanged after the initial reversals at the onset of the medication. This suggest that the drugs restored and stabilized the homeostatic processes for these elements. SOD and GPX, however, showed a mean increase in level at initiation of treatment, which dropped over time and further increased with long time use of the HAART treatment.

Conclusion

In conclusion, this study contributes to knowledge and evidences that treatment with HAART, though effective in reducing viral load of HIV infected patients, further increases the productions of free radicals in the HIV positive subjects on HAART and this is based on the significant increase of the antioxidant enzymes (SOD & GPX) activities found amongst the subjects on HAART treatment as against the HAART-naïve subjects. Also, the study suggest a positive modifications of the electrolytes (Calcium, Phosphorus & Magnesium) as evidenced by the significant increase in the values for Calcium & Magnesium obtained for the HIV positive subjects on HAART as against the HIV positive HAART naïve subjects and lower values for Phosphorus. The knowledge from this study could help guide the way the HAART treated patient in Nigeria are managed, by periodically evaluating their antioxidants profile and introducing antioxidant supplements alongside the drugs.

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