The Effects of Highly Active Antiretroviral Therapy on the Levels of Superoxide Dismutase, Catalase and C-Reactive Protein in HIV Infected Subjects in Nigeria

Faustina Nkechi Osuji1, *, Charles Chinedum Onyenekwe2, Nkeiruka Rose Ukaibe2, Joseph Ebere Ahaneku3

1Laboratory Department, Immaculate Heart Specialist Hospital, Nkpor-Agu, Nigeria
2Department of Medical Laboratory Science, NnamdiAzikiwe University, Nnewi Campus, Nigeria
3Department of Chemical Pathology, Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria

Email address: faustinaosuji@gmail.com (F. N. Osuji), cc.onyenekwe@unizik.edu.ng (C. C. Onyenekwe), nr.ukaibe@unizik.edu.ng (N. R. Ukaibe), cj.ahaneku@unizik.edu.ng (J. E. Ahaneku)
*Corresponding author

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Abstract: The study determined the effects of highly active antiretroviral therapy on the levels of Superoxide dismutase, catalase and C-reactive protein HIV-infected subjects in Nigeria. A total of 50 HIV infected subjects aged 20-69 (39±10) years and 50 HIV seronegative control participants aged 21-56 (35±10) years were recruited for the study. Blood samples were collected at 3 different points: before initiation of HAART, 6 months and 12 months into HAART. The serum levels of C-reactive protein (CRP), catalase, Superoxide Dismutase (SOD), CD4 + T cells and viral load counts were measured in these subjects before HAART initiation and at 6 and 12 months after HAART intake. Standard laboratory methods were used in the analysis of these parameters. The results showed that CRP was significantly increased in HIV infected subjects before commencement of HAART and remained significantly increased after 12 months intake of HAART compared to control participants (P<0.01) respectively. SOD, and CD4 were significantly lower before HAART initiation and after 12 months intake of HAART compared to control participants (P<0.01) respectively. Viral load was significantly reduced after 12 months intake of HAART. There was a negative correlation between the viral load and SOD (r=0.41, P<0.01) and catalase (r=0.47, P<0.01) and a positive correlation between the viral load and CRP (r=0.48, P<0.01) before HAART initiation. After 12 months of HAART catalase showed a significant negative correlation with viral load (r=-0.37, P<0.05) while CRP showed a positive correlation with viral load (r=0.33, P<0.05). The study shows a persistently elevated CRP and reduced SOD and catalase after 12 months intake of HAART. These biomarkers support a central role of inflammation and oxidative stress in HIV pathogenesis.

Keywords: Highly Active Antiretroviral Therapy, HIV, Catalase, SOD, C-Reactive Protein

1. Introduction

Effective Highly Active antiretroviral therapy (HAART) has dramatically improved the life expectancy of persons living with human immunodeficiency virus (HIV). However, even with long term effective HAART, HIV infected persons have persistent low-grade inflammation and immune activation [1]. Levels of acute phase proteins as markers of inflammation usually rise markedly during acute and chronic inflammations. This rise is particularly great for C-reactive protein (CRP) an acute phase protein recognized as an important indicator of inflammatory conditions that are often the consequence of infections [2]. The relationship between CRP and HIV is still unclear, lower levels of CRP have been shown to predict longer survival within HIV infected
individuals [1]. Some studies have observed an association of a single elevated CRP levels with disease progression [3], [4]. An association between higher CRP concentrations, lower CD4+ T cell counts and higher HIV RNA levels has been reported in HIV-infected patients [2]. In addition, regardless of progression to AIDS, HIV infected individuals had a significant increase in CRP over time [5].

The persistent chronic inflammation occurring during HIV infection is also associated with chronic oxidative stress and over utilization of antioxidant defences which may contribute to the loss of immune cells and faster disease progression [6]. Cells are protected against oxidative damage by defence systems. These defence systems include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). Among these SOD plays a central role in the metabolism of reactive oxygen species (ROS) by directly dismutating the superoxide anion radical and production of H2O2 which is scavenged by catalase and GPx [7]. Enzymatic antioxidant defence systems against free radicals are formed by superoxide dismutase glutathione and catalase [8]. In vitro, HIV-1 infection of macrophages resulted in both increased superoxide anion production and elevated SOD mRNA levels, compared with uninfected macrophages. These results indicate that this increase in superoxide anion contributes to the pathogenesis of HIV infection [9]. Several studies have shown clear evidence that oxidative stress may contribute to several aspects of HIV disease, including viral replication [10], even in those treated with antiretroviral therapy [11], this type of oxidant challenge affects the cellular system, and create responses that may be favourable for the replication of HIV [12]. This study therefore determines the level of endogenous antioxidant enzymes SOD and catalase and the inflammatory marker CRP in HIV infected subjects before and during HAART as a possible complementary biomarker for HIV treatment response assessment.

2. Materials and Methods

2.1. Study Population

One hundred subjects were randomly recruited for this study, which consists of 50 HIV infected individuals and 50 HIV negative control individuals. The HIV infected subjects were aged 20-69 years (39±10) years and were recruited before commencement of HAART. The recruitment and study was carried out at Nnamdi Azikiwe University Teaching Hospital Nnewi. The study subjects were followed up for 12 months after commencement of HAART. Blood samples were collected at 3 different points: before initiation of HAART, 6 months into HAART and 12 months into HAART.

A. Before Commencement of HAART: These were individuals confirmed for HIV-1/2 seropositivity. They were eligible for first line HAART. These individuals were started on HAART after sample collection.

6 months into HAART: These were subjects in A above who have taken HAART for 6 months. Blood samples were collected from them exactly after 6 months intake of HAART.

12 months into HAART: These were subjects as in A above who have taken HAART for 12 months. Blood samples were collected from them exactly after 12 months intake of HAART.

B. Control Subjects: The control subjects were HIV seronegative aged (21 – 56) (35±10 years).

2.2. Study Design

The study design was a longitudinal cohort study in which blood samples were collected from study subjects before initiation of HAART, 6 months into HAART and 12 months into HAART. The sampling technique used was simple random sampling technique where the sample size was mathematically calculated.

2.3. Sample Collection

Ten millilitres of fasting blood were drawn by venipuncture from all subjects in the study. The blood samples were aliquoted appropriately into k2-EDTA for CD4+ T cell, and viral load and serum separator tubes (SST) for obtaining clear serum for the determination of SOD, Catalase and CRP. The sera were separated and stored at ≤ -20°C until analyses.

2.4. Methods

2.4.1. Determination of CD4+ T-Cells and CD4+ T-Cells Count by Cyflow SL Green

20μl of whole blood in EDTA anti-coagulant was dispensed into a Partec test tube and 20μl of CD4 PE antibody was added. The reaction mixture was incubated in the dark for 15 minutes. After incubation, 800μl of the already prepared diluted buffer (Xn 0.09% NaN3) was added to each reaction tube and vortexed. The partec tubes containing these reactions were plugged in position in the Cyflow SL Green (Partec Germany), which has already been connected to flow max software, CD4 count template data file and CD4 count instrument. The test was run on the Cyflow for 90 seconds. The results were displayed as histogram and printed. The CD4+ T-Cell count was read off the histogram correcting for the dilution factor.

2.4.2. Viral Load Measurement

HIV-1 RNA quantitation was by Amplicor HIV-1 Monitor (Roche Diagnostics Corporation; Branchburg, New Jersey, USA). The Ultra Sensitive procedure, performed according to the manufacturer’s recommendations, has a measuring range of 20-750,000 RNA copies/mL. During the specimen preparation procedure, the HIV 1/2 viral particles in plasma are concentrated by high speed centrifugation, followed by lysis of the virus particles with a chaotropic agent containing Tris-HCl buffer, 68% Guanidine thiocyanate, 3% Dithiothreitol and < 1% Glycogen. This is followed by precipitation of the HIV RNA with alcohol. A known number of quantitation standard RNA molecules are introduced into
each specimen with the lysis reagent. The HIV Quantitation Standard is carried through the specimen preparation, reverse transcriptase, amplification and detection steps and is used for the quantitation of HIV RNA in each specimen.

2.4.3. Superoxide Dismutase Method

The procedure was as described by the manufacturer of the kit (BioAssay systems, USA) using a clear flat-bottom 96 well plate. Serum samples were diluted 1:5 prior to assay. 20µL SOD standards and samples was added to separate wells of a clear flat-bottom 96-well plate, then 160 µL working reagent (160µL Assay Buffer, 5µL Xanthine and 5µL WST-1) was added to each well plate and mixed thoroughly. 20µL of diluted XO enzyme (1:20 in diluent) was quickly added using a multi-channel pipette and mixed. The OD was read immediately at 440 nm (OD260). The plate was then incubated at 60 minutes in the dark at room temperature after incubation it was read at OD 440nm again (OD60). For each standard and sample calculate ∆OD60 = OD60 - OD260. A standard curve was plotted and used to determine the sample concentration.

2.4.4. Serum Catalase Measurement

The procedure was as described by the manufacturer of the kit (BioAssay systems, USA) using a clear flat-bottom 96 well plate. 10µL of subjects sample and control was placed into wells of the 96-well plate. One sample blank well was prepared that contains only 10µL of Assay Buffer. A dd 90µL working standard containing 1µmole of H2O2 and 10µL of diluent was added to each well plate and mixed thoroughly. 20µL of diluted XO enzyme (1:20 in diluent) was added to each well and incubated for 10 minutes. The absorbance was read at 570nm. H2O2 standard curve was prepared and used to determine the concentration of catalase in the sample. One unit is the amount of catalase that decomposes 1µmole of H2O2 per min at pH 7.0 and room temperature.

2.4.5. Statistical Analysis

The results were presented as mean ± standard deviation. Differences between the results of the control subjects and those of HIV positive subjects before commencement of HAART, 6 months and 12 months into HAART were analyzed using student’s t test and ANOVA. Pearson correlation was employed in analyzing the relationship between viral load count and the SOD, catalase and CRP. The p value of 0.999 is considered statistically significant. Differences were considered significant at P<0.05.

2.4.6. The Antiretroviral Drug Combination for the HIV Subjects

a) Tenofovir 300mg, Lamivudine 300mg, Efavirenz 600mg (TENOLAM E). This is a ‘first line’ drugs combined in a single pill.

b) Dosage and Administration: Adults and adolescents weighing ≥ 30kg: 1 tablet daily in the evening. There was no adverse drug reactions reported by the subjects during therapy and there was no interruption of therapy by the subjects. The drug combination, dosage and administration are according to WHO preferred initial regimen for adults and adolescents as of June 2013, which include: tenofovir + lamivudine + efavirenz (i.e 2 NRTIs and a NNRTI). (WHO, 2013). This first line drugs can only be changed for a patient experiencing adverse drug reaction or in cases of drug resistant which is indicated by high viral load count after 6 months of therapy. None of the subjects showed adverse drug reaction or drug resistant.

3. Results

Table 1 shows significant differences in serum levels of Superoxide Dismutase (U/mL) in HIV infected subjects before commencement of HAART (0.12±0.01), 6 months into HAART (0.54±0.08), 1 year into HAART (1.23±0.09) and control subjects (2.45±0.23) (F=12.50, P<0.01). Between group comparison showed that there was a significant mean difference before commencement of HAART compared with 6 month and 1 year into HAART respectively(P<0.01 in each case). SOD level was significantly lower before commencement of HAART compared with 1 year into HAART (P<0.05) and compared with control subjects (P<0.01). See table 1.

Serum catalase was significantly different in HIV infected subjects before commencement of HAART (1.87±0.36), 6 months into commencement of HAART (2.76±0.83), 1 year into HAART (3.05±1.13) and control subjects (3.91±1.22), (F=10.63, P<0.01). Catalase level was lower before commencement of HAART compared respectively with 6 months and 1 year into HAART (P<0.05 in each case). There was also a significant mean difference after 1 year into HAART compared with control subjects (P<0.01). See Table 1.

Serum C-Reactive Protein (CRP) (ng/ml), was significantly different in HIV infected subjects before commencement of HAART (2.86±0.10), 6 months into HAART (1.40±0.62) , 1 year into HAART (1.36±0.63) and in control subjects (0.83±0.39) (F=18.15, P<0.05). CRP serum level was higher before commencement of HAART compared respectively with 6 months and 1 year into HAART (P<0.01) respectively. There was also a significant higher serum CRP during 1 year into HAART compared with control subjects (P<0.05). See Table 1.

### Table 1. Serum Levels of superoxide Dismutase (U/mL) Catalase (U/L) and CRP (ng/ml) in HIV infected subjects before commencement of HAART, 6 months and 1 year into commencement of HAART (±SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U/mL)</th>
<th>Catalase (U/L)</th>
<th>CRP (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before commencement of HAART [a] n = 50</td>
<td>0.12±0.01</td>
<td>1.87±0.36</td>
<td>2.86±0.10</td>
</tr>
<tr>
<td>6 Months into HAART [b] n = 50</td>
<td>0.54±0.08</td>
<td>2.76±0.83</td>
<td>1.40±0.62</td>
</tr>
</tbody>
</table>
Table 2. CD4+ T cell count (/µl) and viral load (Log10 copies/ml) in HIV infected subjects before commencement of HAART. 6 months and 1 year into HAART.

<table>
<thead>
<tr>
<th>Group</th>
<th>CD4+ T cells /µl</th>
<th>Viral Load(Log10 copies /ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before commencement of HAART [a] (n= 50)</td>
<td>152±67</td>
<td>5.45±0.37</td>
</tr>
<tr>
<td>6 Months into HAART [b] (n= 50)</td>
<td>378±162</td>
<td>2.70±0.63</td>
</tr>
<tr>
<td>1 Year into HAART [c] (n= 50)</td>
<td>522±160</td>
<td>0.18±0.04</td>
</tr>
<tr>
<td>Control [d] (n= 50)</td>
<td>900±245</td>
<td>-</td>
</tr>
</tbody>
</table>

F Value | 27.73 | 13.09 |
P Value | 0.00 | 0.00 |

[a] Vs [b] | P<0.05 | P<0.05 |
[a] Vs [c] | P<0.05 | P<0.05 |
[a] Vs [d] | P<0.05 | P<0.05 |
[b] Vs [c] | P<0.05 | P<0.05 |
[b] Vs [d] | P<0.05 | P<0.05 |
[c] Vs [d] | P<0.05 | - |

n, number of participants; HAART, Highly Active Antiretroviral Therapy; Vs, Versus.

A significant correlation was observed between viral load and SOD (r=0.41, P<0.05) and between viral load and catalase (r=0.47, P<0.05) respectively before commencement of HAART. There was also significant correlation observed between CD4+ T Cells and SOD (r=0.36, P<0.05) and between CD4+ T cells and catalase (r=0.35, P<0.05) before commencement of HAART. A significant negative correlation was seen between viral load and CRP (r=-0.48, P<0.05) before HAART initiation. See table 3.

Table 3. Correlation between Viral load (Log10 copies/ml), Catalase (U/L) and SOD (U/mL) in HIV infected before commencement of HAART.

<table>
<thead>
<tr>
<th>parameters</th>
<th>n</th>
<th>r value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral load VsSOD</td>
<td>50</td>
<td>-0.41</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Viral Load VsCatalase</td>
<td>50</td>
<td>0.47</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Viral Load Vs CRP</td>
<td>50</td>
<td>0.48</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Viral load Vs CD4+ T cells</td>
<td>50</td>
<td>-0.72</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CD4+ T cells Vs SOD</td>
<td>50</td>
<td>0.36</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CD4+ T cells Vs Catalase</td>
<td>50</td>
<td>0.35</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CD4+ T cells Vs CRP</td>
<td>50</td>
<td>-0.52</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Correlation was analyzed by Pearson’s correlation.
SOD, Superoxide Dismutase; CRP, C-Reactive Protein; n, number of participants; Vs, Versus.
There was no significant correlation observed between viral load and SOD in HIV infected subjects after 1 year into HAART, but there was a significant negative correlation between viral load and catalase (r = -0.44, P<0.05), in HIV infected subjects 1 year into HAART. There was also a significant correlation observed between CD4 + T Cells and SOD (r=0.41, P<0.05) and CD4 + T Cells and catalase (r=0.38 P<0.05) in HIV infected subjects 1 year into HAART. See table 4.

4. Discussion

The present study revealed a decrease in the level of SOD in serum of HIV infected subjects than in control group. This decrease was more marked before HAART initiation. Such decrease may be due to detoxification of released ROS (superoxide anion). In HIV infected patients, this detoxification results in the formation of hydrogen peroxide which inactivates SOD. Therefore the accumulation of hydrogen peroxide may be one of the explanations for decreased activity of SOD in these patients [13].

The catalase levels were found to be significantly decreased in HIV infected before HAART intake and at 6 months intake of HAART compared to control participants. This decrease in catalase accounts for production of high ROS, suggesting that increased oxidative stress might be attributed to the deficiency of antioxidant defence system. This deficiency in HIV infected subjects may be due to increased utilization of catalase. The increased activity found after 1 year of therapy may be as a result of less production of ROS and free radicals due to progressive increase in CD4 + T cells and decrease in viral load after 1 year of therapy. Several reports showed lower activities of catalase and other antioxidants in HIV positive non-treated and those treated with HAART when compared with control subjects [9, 14-16]. Following initiation of HAART for 1 year, the activities of catalase and SOD increased, which show progressive treatment and clearance of ROS by HAART.

Correlation results showed positive correlation between CD4 T cells and SOD before commencement and during HAART and between CD4+ T cell and Catalase before commencement of HAART. This could suggest a reduced oxidative stress environment and increased SOD/Catalase during HAART compared to before commencement of HAART, though both maintained reduced endogenous antioxidant enzymes in comparison with control subjects. The study concurs with reports provided by [17]. It can be envisaged that decreased oxidative stress is a component of the dynamic process of immune reconstitution in some subjects with HIV, rather than a more direct effect of drug. We could presume that SOD and catalase are protective in nature as can be attested to in the correlation results. Inversely the observed decrease in Catalase and SOD activities before commencement of HAART could be attributed to rapid depletion of these endogenous enzymes by high level of circulating chemically reactive species associated with the HIV untreated patients. The decrease in SOD and Catalase activities in HIV infected subjects before commencement of HAART could account for the production of high ROS in these subjects. This is in line with various studies which suggested that an increased oxidative stress might be attributed to the deficiency of antioxidants defence system [18, 19]. This deficiency of antioxidants in HIV infected subjects before commencement of HAART may be due to increased utilization of antioxidant enzymes such as superoxide dismutase and catalase. It has been reported that HIV patients are constantly under oxidative stress evidenced by the low levels of SOD and increased levels of cortisol [20].

HIV infection is characterized by severe immunodeficiency, a consequence of numerical and functional CD4 + T cell depletion [21]. The CD4+ T cell count is an important biomarker for HIV progression; our results showed that CD4 + T cells count were significantly lower in HIV infected subjects. A statistically significant recovery of CD4+ T cell count was demonstrated 6 months and 12 months after starting HAART. The CD4+ T cells count increased from 152.84 /µl before commencement of HAART to 378.90 /µl after 6 months of HAART and to 522.58 /µl after 1 year into HAART. [22] had earlier reported a significant increase in CD4+ T cells count between 3 months to 6 months of HAART in their study. This clearly demonstrated general immune reconstitution after commencement of HAART. The subjects also attained viral load undetectability after 1 year of therapy, the above findings are consistent with an improvement in immune-virological parameters [23].

The level of CRP in HIV infected subjects before commencement of HAART was significantly higher and the increase was sustained up to 1 year into HAART. This observation may indicate the chronic inflammatory state of HIV infected patients which will definitely enhance their state of oxidative stress. This chronic inflammation may reduce when appropriate HAART is initiated as seen by progressive reduction in CRP when HAART was initiated. The results also show that CRP levels correlated positively with viral load and negatively with CD4+ T cells levels, both before commencement of HAART, 6 months and 1 year into HAART.

Levels of CRP were associated with HIV disease progression independent of CD4 lymphocyte counts and HIV RNA levels [24]. In addition, regardless of progression to AIDS, HIV-infected individuals had a significant increase in CRP over time. Levels of acute-phase proteins
as markers of inflammation usually rise markedly during acute and chronic infections. This rise is particularly great for C-reactive protein (CRP), an acute-phase protein recognized as an important indicator of inflammatory conditions that are often the consequence of infection. This suggests a potential role of CRP in monitoring the clinical course of HIV infected individuals. Use of HAART for 1 year was not significantly associated with a decrease in CRP when compared with control subjects, indicating that HAART intake for 1 year did not have a profound effect on CRP concentration. The gradual decrease in CRP during therapy suggests that CRP concentrations may have a prognostic values, such measurement cannot replace CD4+ T cell counts and HIV RNA levels for monitoring HIV infected subjects. [24] suggest that CRP appears useful for diagnosis and monitoring of intercurrent infection in HIV-1 antibody-positive patients. In HIV-1 antibody-positive patients without intercurrent infection, CRP values are high compared to levels in the general population which possibly reflect a sustained acute-phase response as a consequence of HIV infection.

C-reactive protein is a sensitive marker of inflammation and tissue damage which may trigger apoptosis. The hallmark of HIV infection is cellular CD4 immunodeficiency. Different agents may trigger apoptosis of CD4 T cells including viral protein (gp 120, Tat) inappropriate secretion of inflammatory cytokines by activated macrophages. The inflammatory environment leads to increased CRP. Since oxidative stress can also induce apoptosis, it can be hypothesised that such a mechanism could participate in CD4 T cells apoptosis observed in HIV/AIDS and this can lead to progression of HIV disease in the absence of HAART. Increased levels of C-reactive protein are an early marker of opportunistic infections in HIV. [25],[26]

5. Conclusions

Our data show that measurement of serum levels of SOD, Catalase and CRP before initiation of HAART may be useful in risk stratification of patients who are about to initiate HAART. The presence of elevated levels of these biomarkers before initiation of HAART as well as persistently significant high levels after 1 year of HAART could assist in selecting patients who could benefit from closer monitoring or additional workup to unveil occult infections. In addition, these biomarkers support a central role of inflammation and oxidative stress in HIV pathogenesis. On the other hand, in developing countries such as Nigeria, the ever-growing incidence of HIV infection has placed a huge burden on their frail economy, so there is a growing need for simplifying HIV treatment protocols and for having cheaper alternatives for monitoring disease activity. Serial monitoring of CRP and antioxidant levels could identify individuals who are at highest risk of HIV disease progression and those that may benefit from antioxidant and anti-inflammatory therapies.

Abbreviations

HAART: Highly Active Antiretroviral Therapy
HIV: Human Immunodeficiency Virus;
CRP; C-reactive protein
mRNA: Messenger Ribonucleic acid
NAUTH: Nnamdi Azikiwe University Teaching Hospital
SOD: Superoxide dismutase
SST: Serum separator tubes
XO: Xanthine oxidase
OD: Optical Density

Declarations

I declare that this research is original and has not been submitted for publication in another journal and that the results presented above are true and correct to the best of my knowledge.

Ethics Approval and Consent to Participate

The subjects gave informed consent while the study design was approved by the Nnamdi Azikiwe University Teaching Hospital (NAUTH) Ethics Committee. (Ref: NAUTH/CS/66/VO.4/61).

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they do not have any conflict of interest.

Funding

Funding information is not applicable. No funding was received.

Author’s Contributions

FN conducted the laboratory and statistical analysis under the supervision of CC. All Authors revised and edited the manuscript. Final version of the manuscript was approved by all authors.

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