Evaluation of Oxidative Stress in AIDS Pediatric Patients

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SUMMARY. Infection by human immunodeficiency virus (HIV) continues to increase worldwide. The progression of the disease in children may be rapid. Oxidative stress is well documented in adult HIV/AIDS patients. Viral Tat protein plays a role in the intracellular generation of reactive oxygen species thus increasing apoptotic index, and depleting CD4⁺ T lymphocytes. The aim of this study was to investigate an extensive array of redox status indices: glutathione (GSH), malondialdehyde (MDA), peroxidation potential, total antioxidant status, glutathione peroxidase (GPx), superoxide dismutase (SOD), Total hydroperoxide (TH) and DNA fragmentation in AIDS pediatrics patients compared to healthy subjects. Blood samples from 11 pediatric patients and 22 healthy subjects were tested by spectrophotometric techniques in order to measure oxidative stress indices. Both a reduction of GSH levels and an increase in MDA and TH levels were detected in the plasma of AIDS patients. These patients also showed an increase of DNA fragmentation in lymphocytes as well as a reduction of GPx and an increase in SOD activity in erythrocytes. Relatively to the control group, AIDS patients had differences in global indices of total antioxidant status. These results contribute to the evidences that substantial oxidative stress occurs during AIDS pediatric infection.

RESUMEN. “Evaluación del Estrés Oxidativo en Pacientes Pediátricos con SIDA”. La infección por el virus de la inmunodeficiencia humana (VIH) continúa aumentando a nivel mundial. La progresión de la infección en niños es rápida. El proceso de estrés oxidativo asociado a la infección en adultos está bien documentado. La proteína viral Tat juega un papel reconocido en la generación intracelular de especies reactivas de oxígeno, evento este que repercute en el incremento del índice de apoptosis y en la disminución de los linfocitos T CD4⁺. El objetivo del presente trabajo fue evaluar un extenso grupo de marcadores del estado redox tales como: glutación (GSH), malondialdehído (MDA), potencial de peroxidación (PP), estado antioxidante total (TAS), glutación peroxidasa (GPx), superóxido dismutasa (SOD), hidroperóxidos totales (TH) y % de fragmentación del ADN, en pacientes pediátricos comparados estadísticamente con individuos sanos. Muestras de sangre de 11 pacientes SIDA pediátricos y de 22 individuos sanos fueron utilizadas para realizar las mediciones de los marcadores del estado redox por técnicas espectrofotométricas. En el plasma de los pacientes SIDA pediátricos se detectó un nivel bajo de GSH, así como niveles altos de MDA y TH. Además se observó un mayor porcentaje de fragmentación del ADN en linfocitos, actividad enzimática de GPx disminuida y actividad mayor de SOD en eritrocitos en comparación con el grupo de individuos sanos. Se observaron diferencias significativas en cuanto a los índices del estado antioxidante global. Estos resultados corroboran las evidencias que el estado de estrés oxidativo se manifiesta durante la infección SIDA en pacientes pediátricos.

INTRODUCTION

Human Immunodeficiency virus (HIV) infection is a worldwide problem and HIV/AIDS patients suffer from several opportunistic infections. The hallmark of HIV infection is cellular CD4 immune deficiency. Different agents appear may trigger apoptosis in CD4⁺ T cell, including: viral protein (i.e. gp 120, Tat), inappropriate secretion of inflammatory cytokines by activated macrophages (i.e. tumor necrosis factor alpha (TNF-α) and toxins produced by opportunistic microorganisms. Since oxidative stress can also induce apoptosis, it can be hypothesized that such a mechanism could participate in CD4⁺ T cell apoptosis observed in AIDS. Oxidative stress results from the imbalance between reactive oxygen species (ROS) production and its inactivation 1,2.

Under most circumstances, oxidative stress is deleterious to normal cell functions. An emerging view, however, is that, within certain limits, cellular redox status is a normal physiological

KEY WORDS: AIDS, CD4⁺ T lymphocytes count, HIV, Oxidative stress.

PALABRAS CLAVE: Estrés oxidativo, Conteo de Linfocitos T CD4+, SIDA, VIH.

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variable that may elicit cellular response such as transcriptional activation, proliferation or apoptosis. Exposure to oxidants challenges cellular systems and their responses may create conditions that are favorable for the replication of viruses such as HIV.

In HIV-infected patients increased oxidative stress has been implicated in increased HIV transcription through the activation of nuclear factor κB (NF-κB). NF-κB is bound to factor IκB in the cytoplasm in its active form, but various factors, such as TNF-α and ROS can cause the release of NF-κB from factor IκB, and NF-κB translocates to the nucleus and binds to DNA. Glutathione is a major intracellular thiol, which acts as a free radicals scavenger and is though to inhibit activation of NF-κB. NF-κB is involved in the transcription of HIV-1. Thus, ROS may potentially be involved in the pathogenesis of HIV infection through direct effects of cells and through interactions with NF-κB and activation of HIV replication.

Recently, it has been demonstrated that viral Tat protein liberated by HIV-1-infected cells interferes with calcium homeostasis, activates caspases and induces mitochondrial generation and accumulation of ROS, all being important events in the apoptotic cascade of several cell types. CD4+ T cell subset depletion in HIV/AIDS patients is the most dramatic effect of apoptosis mediated by redox abnormalities.

Some micronutrients play essential roles in maintaining normal immune function and may protects immune effectors cells from oxidative stress. For pediatric HIV patients is particularly important to identify metabolic alterations and deficiencies and determine whether the supplementation will improve clinical outcome. This requires realistic and sustainable healthcare interventions in terms of costs, technology transfer and independence from sophisticated monitoring requirements.

Others group reported that serum MDA levels are increased in HIV infected patients' children, when compared with control and proposed the use of these levels as additional criteria for the clinical follow up of pediatric patients.

The markers for evaluate HIV progression used in clinical practice: CD4+ T lymphocyte count, viral load and the classification proposed by the CDC 1994, show different shortcomings when applied to pediatric population, in view of the immaturity of the immune system. These facts make it necessary to seek for additional analytical markers for the clinical management of HIV infected children.

The aim of the present work was to study the status of an extensive array of oxidative stress indices that would permit to examine the stress target which cause damage to biomolecules in AIDS pediatric patients. For this purpose we compared the blood levels of these markers in AIDS patients to those of healthy aged-matched control.

**MATERIALS AND METHODS**

**Subjects and blood collection**

Blood and serum samples from 11 pediatric patients infected with HIV and aged 1 to 13 years were used. These group patients constituted the total Cuban AIDS children at the moment of research. Control subjects were 22 sex- and age-matched healthy, HIV seronegative individuals. Subjects gave informed consent to take part in the study after verbal and written explanation of the methods involved. Study procedures were reviewed and approved by the Institute "Pedro Kouri" (Hospital) Committees for Research on Human Subjects. Blood samples were obtained after at least 12-hour fasting. Serum was stored at -70 °C until analysis.

Seropositive individuals had a reactive enzyme-linked immunosorbent assay for HIV (ELISA-UNI FORM VIH I/II plus O, Organon Technica), which was confirmed by Western Blot analysis (DAVIH BLOT VIH-I, DAVIH Lab). All patients and control subjects had no family history of coronary heart disease. None of the patients suffered from diarrhea, defined as more than two loose stools per day for at least 1 month.

**Flow cytometry analysis**

Whole blood T lymphocyte subsets CD3+/CD4+ were analyzed with the use of Becton Dickinson (BD) lysing solution. For the CD4+ T lymphocyte subsets Tri Test TM CD3 CD45 CD4 was used. Analysis were performed on a FACScan flow cytometer (Becton Dickinson Immunocytometry System) using the MULTISSET program and standard methodology. The results are expressed as a percentage of lymphocytes.

**Biochemical measurements**

Glutathione Concentration

Serum reduced glutathione (GSH) was analyzed with the method described by Sedlak and Lindsay (1968). All of the non-protein sulfhydryl groups are in the form of reduced glutathione. 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) is a disulfide chromogen that is readily reduced by sulfhydryl compounds to an intensely yellow...
compound. The observance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration. GSH (Sigma, St. Louis, M.O., USA) was used to generate standard curves \(11-13\). Values were expressed as mg/L.

**Malondialdehyde Concentration**

Malondialdehyde (MDA) concentrations were analyzed with the LPO-586 kit obtained from Calbiochem (La Jolla, C.A., USA). In this assay, stable chromophore production after 40 min of incubation at 45 °C is measured at a wavelength of 586 nm by Pharmacia Spectrophotometer. Freshly prepared solutions of malondialdehyde bis [dimethyl acetal] (Sigma, St. Louis, M.O., USA) assayed under identical conditions were used as reference standards. Concentrations of MDA in serum samples were calculated using the corresponding standard curve and values were expressed as nmol/mL \(14,15\).

**Peroxidation Potential (PP)**

For the determination of the susceptibility to lipid peroxidation, serum samples were incubated with a solution of cupric sulfate (final concentration of 2 mM) at 37 °C for 24 h. The PP was calculated by subtracting the MDA concentration at time 0 from the one obtained at 24 h \(16\).

**Total hydroperoxide (TH)**

TH was measured by Bioxytech H$_2$O$_2$-560 kit Cat. 21024 (Oxis internacional Inc. Portland, USA). The assay is based on the oxidation of ferrous ions to ferric ions by hydroperoxides under acidic conditions. Ferric ions bind with the indicator dye xylenol orange (3,3’-bis(N,N-di(carboxymethyl)-aminomethyl)-o-cresol sulphone-phatein, sodium salt) to form a stable colored complex which can be measured at 560 nm. Values were expressed as mol/mL \(17\).

**Total antioxidant status (TAS)**

For TAS quantitation Randox Ltd. (Diamond Road, Crumlin, U.K) Kit Cat. No. RS505. In brief, GPx catalyses the oxidation of GSH by cumene hydroperoxide. In the presence of glutathione reductase and NADPH the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP$^+$. The decrease in absorbance at 340 nm is measured \(18\). Values were expressed as U/g Hb.

**Superoxide dismutase (SOD)**

Evaluation of SOD activity was determined using Randox Ltd. (Diamond Road, Crumlin, U.K.) Kit Cat. No.SD125. In brief, the method employs xanthine and xanthine oxidase to generate superoxide radicals, which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase activity is then measured by the inhibition degree of this reaction \(18\). Values were expressed as U/g Hb.

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company (St. Louis, M.O., USA).

**Statistics**

Data are expressed as means ± SEM. The AIDS group was compared with the seronegative control subjects by using independent t test, two tailed. The minimal level of significance was identified at p<0.05. The SPSS software package was used for all statistical analyses \(20\).

**RESULTS**

The AIDS group consisted of 11 individuals with symptomatic HIV infection (mean CD4$^+$...
Table 1. Characteristics and percentages of CD4+ and CD8+ in controls and in AIDS pediatric patients. Values from groups are significantly different (p<0.05) within the same set. Control: seronegative individuals. AIDS: patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (years)</th>
<th>Sex</th>
<th>CD4⁺ Mean (Intervals)</th>
<th>CD8⁺ (%) Mean (Intervals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22</td>
<td>1-13</td>
<td>Female</td>
<td>8</td>
<td>41 (37-45)</td>
</tr>
<tr>
<td>AIDS</td>
<td>11</td>
<td>1-13</td>
<td>Male</td>
<td>14</td>
<td>15 (9-21)</td>
</tr>
</tbody>
</table>

13%; range 5-17%). Risk factor for HIV infection was vertically transmission (100%). At the time of sampling all patients (100%) were receiving combination antiretroviral therapy. The control group was composed of 22 healthy, seronegative individuals recruited from Pediatric consult of hospital. Control subjects had no acute or chronic illness and were not taking any medications or nutritional supplements.

There were no significant age differences (p>0.05) between AIDS patients and control subjects, respectively. AIDS patients had significantly (p<0.05) lower CD4+ T lymphocyte count (%) compared to control subjects (Table 1).

Serum GSH levels were significantly (p<0.05) lower in AIDS patients. Lipid peroxidation determined from MDA serum concentration was significantly (p<0.05) higher in the AIDS group compared to control subjects (Fig. 1 A, B). Leukocyte DNA fragmentation (%) and serum levels of TH were significantly (p<0.05) increased in AIDS patients (Fig. 2 A, B).

The activities of the antioxidant enzymes GPx and SOD in erythrocytes are shown in Fig. 3. GPx activity was significantly (p<0.05) decreased in AIDS patients while SOD activity was significantly (p<0.05) increased in AIDS subjects. PP and TAS assayed serum antioxidant capacity. The former is a marker of serum susceptibility to lipid peroxidation. AIDS patients had TAS levels that were generally lower than controls and PP significant increases were noted in AIDS group (Fig. 4).

DISCUSSION

Human monocyte-derived macrophages are involved in a variety of pathological events in HIV infection the hallmark of which is immunodeficiency with progressive CD4+ T lymphocyte depletion 2. Our AIDS pediatric patients ex-
hibited the characteristic loss of CD4+ T cells (Table 1). Even if the exact cause of this loss of CD4+ T cells is unknown, the most widely accepted hypothesis is that HIV primes the cell to apoptotic death 20.

HIV infection cause a chronic inflammation as shown by high plasma levels of inflammatory cytokines and production of ROS in seropositive individuals 2. The oxidative stress occurring at HIV disease was shown by a decrease of the major antioxidant molecules. The observed dramatic reduction in GSH plasma levels of patients is shown in Fig. 1. By other hand GSH is a cofactor of GPx enzyme. AIDS pediatric patients showed a decrease of the GPx activity with the same behavior of GSH depletion (Fig 3) suggesting the participation of both anti-oxidative mediators in the disruption of the redox balance. In line with these findings there was an increase of TH which is able to promote DNA damage and others injurious effects. Plasma sulfhydryl groups have an important protective function because they act as antioxidants. In fact, their decrease has been associated to oxidation as suggested by the presence of superoxide anion, hydrogen peroxide and hydroxyl radical 21,22. The presence of oxidized SH groups can also contribute to potentiate oxidative stress by the additional generation of ROS independently from specific pH levels, presence of metals and concentration of oxidized SH.

An increase in plasma concentration of the byproduct of lipid peroxidation (MDA) was also detected in AIDS pediatric patients (Fig. 1). This increase is consistent with the finding of other studies that showed oxidative stress in HIV pediatric patients as evidenced by MDA concentration 23,24. The similar behavior between MDA, TAS and PP (Fig. 4) suggest the role of lipid peroxidation in the loss of redox cellular status in HIV patients. The observed increase of oxidative stress processes in these patients resulting from cytotoxic products may modify proteins and DNA by addition reactions 25.

Other experiments suggest that lipid peroxidation is much more important in the asymptomatic stage rather than in AIDS. An explanation for this may relate to the depletion of neutrophils which occurs in the late disease stages and which may be influenced by the treatments used by patients 6, 26. According to our results, however, treatment seems to be relevant since 100% of AIDS pediatric patients were receiving antiretroviral therapy. Thus, increased MDA values patients observed in our study may be a consequence of the multifactorial nature of the redox system.

Peroxides serve as a source for hydroxyl or peroxyl reactive radicals who can interact with cellular components inducing cell damage potentially leading to cell death 21. The increase of TH observed in AIDS patients (Fig. 2) emphasizes the higher oxidative stress, which occurs during HIV infection. It should also be noted that peroxides and aldehydes generated are not only passive markers of oxidative stress, but also cytotoxic products 27. It is thus important to evaluate the role of these oxidative products in lymphocyte death. Nuclear DNA fragmentation is a biochemical hallmark of apoptosis 28 and its increase in AIDS pediatric patients (Fig. 2). It is consistent with the hypothesis proposed in 1991 by several groups that reported an association of HIV-infected T cells lost with DNA fragmentation and apoptotic cell death.

Antioxidant enzymes levels are sensitive to oxidative stress. Both increased and decreased levels have been reported in different disease states in which an enhancement of oxygen species is a cause or a consequence of the disease. Fig. 3 shows a significant reduction of GPx activity and an increase of SOD activity in erythrocytes of HIV-infected patients. Under this condition the detoxification capacity of hydroperoxides is reduced and this is probably connected to the high levels of TH detected in pediatric patients (Fig 2A). Several kinds of molecules contribute to the antioxidant capacity of plasma. The possible interaction among different antioxidants in vivo could also render the measurement of any individual antioxidant less representative of the overall antioxidant status 29. PP and TAS are used as total antioxidant capacity marker in plasma. There is experimental evidence that different metabolic events that oc-

![Figure 4](image-url)
cur as a consequence of HIV infection directly influence the consumption of antioxidant components thus contributing to the increase of oxidative stress.

The alteration on PP and TAS in HIV patients point to the multifactorial feature of this infection. Some authors consider oxidative stress not as an epiphenomenon, but rather as having a central role in HIV disease. Authors 2, 25, 30-32.

This study provides evidences of oxidative damage to different molecules in AIDS pediatric patients. The combination of redox indices that could be most useful based on this study is MDA, SOD, PP and TAS. The similar behavior was detected in GSH, GPx and TH. These, together with results pointing to the ROS-mediated activation of the virus, strongly argue in favor of the benefits of both adequately nutrition and early antioxidant therapy. The indicators evaluated could be contributing to an integral overview in AIDS pediatric infection; in addition, further studies may be justified to evaluate the role of ROS as indices of treatment efficacy.

Acknowledgements. We gratefully acknowledge the support from RANDOX (U.K.).

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