Oxidative Stress in HIV/AIDS Patients in Mumbai, India

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Abstract

Background: Viral and host factors are responsible for oxidative stress in HIV disease, which in turn activates the replication of HIV provirus by various pathways. Oxidizing stress is a pathologic phenomenon resulting from imbalance between the system producing active oxygen species and those defending the organism. The present study was aimed to assess oxidative stress markers in HIV patients.

Methods: The study included 65 HIV sero-positive patients attending OPD in a tertiary care hospital in Mumbai. 30 age and sex matched healthy volunteers served as controls. Patients were categorized on the basis of their absolute CD4 counts into 3 groups - Group-1 (<200 CD4 cells/mm3), Group-2 (200–499 CD4 cells/mm3), and Group-3 (>500 CD4 cells/mm3). Pro-oxidant markers like the Thiobarbituric Acid Reactive Species (TBARS), Nitric Oxide, and Advanced Oxidation Protein Product (AOPP) were studied from serum, while generation of Superoxide Anion in the PBMC's was assessed. Antioxidant parameters including enzymes like Catalase (CAT); Superoxide Dismutase (SOD), Glutathione-S-Transferase (GST) and protein thiols were also assessed.

Results: Levels of pro-oxidants were significantly increased whereas antioxidant defense markers were significantly impaired in the HIV seropositive group. The pro-oxidants exhibited a negative correlation when compared with CD4 counts where as antioxidant markers showed a positive correlation.

Conclusion: Severely impaired antioxidant machinery leads to higher levels of pro-oxidants in the system. These in turn contribute negatively to the prognosis of HIV/AIDS. Very few comprehensive assessments have been made correlating oxidative stress and HIV in Indian population. This study may therefore generate important leads in surrogate prognostic markers for while assessing simple biochemical parameters. Also, the role of anti-oxidants may be investigated in the management of HIV/AIDS in Indian population.

Keywords: HIV; Oxidative Stress; Pro-oxidant; Anti-oxidant.

Abbreviations: AIDS: Acquired Immuno Deficiency Syndrome; AOPP: Advanced Oxidation Protein Product; CAT: Catalase; F-SH: Free Thiols; GST: Glutathione S-transferase; HIV: Human Immuno Deficiency Virus; MDA: Malondialdehyde; NF-kB: Nuclear Factor kappa beta; NO: Nitric Oxide; OPA: Ortho Phosphoric Acid; P-SH: Protein Thiol; ROS: Reactive Oxygen species; SOA: Superoxide Anion; SOD: Super Oxide Dismutase; TBARS: Thio Barbituric Acid Reactive Species; T-SH: Total Thiols.

Introduction

Human immunodeficiency virus (HIV) infection is a worldwide problem and HIV/AIDS patients suffer from several opportunistic infections that occur because of poor immune system function. The hallmark of HIV infection is cellular CD4 immunodeficiency. 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 microorganism. 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 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 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. A variety of enzymatic (superoxide dismutase, catalase, glutathione peroxidase etc) and non enzymatic antioxidants

present in human serum become insufficient to circumvent the HIV-1 replication secondary to cellular ROS production (superoxide anion. hydroxyl radical, hydrogen peroxide) by the pro-oxidant effect of inflammatory cytokines or poly morphonuclear leukocyte activation [3]. In HIV-infected patients increased oxidative stress has been implicated in increased HIV transcription through the activation of nuclear factor kB (NF-κB). NF-kB 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 (GSH) is one of the major intracellular thiol, which acts as a free radical scavenger and also is thought to inhibit activation of NF-κB [4]. NF-κB is involved in the transcription of HIV-1. Thus, this shows 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 [5]. The present study was aimed to assess oxidative stress markers in HIV/AIDS patients.

Materials and Methods

All studies on Human Volunteers were approved by the Institutional Ethics Committee of Seth GSM College& KEM Hospital vide letter no.EC/67/2011

Study groups

The study population consists of 65 HIV seropositive patients, as determined by patients' history were included in the study. Patients belonging to both sex i.e. male and female of age of 18 years and above included. For the diagnosis and confirmation of HIV infection, we followed the National AIDS Control Organization (NACO) recommendations for HIV testing all the patients were subjected to detailed history taking and clinical examination. Informed consent of the patients was taken before testing. Individuals were classified on the basis of their absolute CD4+T lymphocyte counts. Patients were separated into three groups- Group 1: <200 cells mm−3; Group 2: 200–499 cells mm^{-3}; and Group 3:≥500 cells mm^{-3}. 30 individuals were selected on the basis of their past history whether they had any infection or not from work site population they are belonging to both sex i.e. male and female of age between 20 years to 40 years. Exclusion criteria: Individuals below 18years old and pregnant women were excluded from the study.

Blood collection

Ten milliliters of whole blood from HIV sero positive patients and healthy volunteers were collected in EDTA and plain vacuum tubes each and was used immediately for the determination. The serum was separated from plain vacuum tube, aliquoted and stored at -20°C and used for the following assays.

Total Sulfhydryl groups (Total-SH), Protein bound Sulfhydryl groups (Protein-SH) and free Sulfhydryl groups (Free-SH)

Serum Total-SH, Protein-SH and Free-SH were determined with slight modification by method described by Ellman and Sedlak and Lindsay [6,7]. Concentration of protein and non-protein SH groups was measured colorimetrically with modified Ellman method in blood serum. To 445µl of PBS buffer pH 7.4, 25 µl of 2 mM of dithionitrobenzoic acid (DTNB) and 50 µl of standard or sample were added. Tubes were centrifuged at 15,000 rpm for 10 minutes and absorbance was measured at 412 nm against blank with DTNB. For Free thiols, in similar way 50 µL of serum was precipitated with 50µl of 50 % Trichloro acetic acid. The tubes were centrifuged at 15,000 rpm and the supernatant was again re precipitation with 50 µl of 50 % of TCA. 195 µL of PBS buffer pH 7.4 with 50 µl of supernatant and 25 µl of 2 mM DTNB was added and absorbance was measured at 412 nm. Reduced GSH was used as standard. For obtaining the Total or free thiols the value was calculated from standard graph and the intercept obtain. To calculate the protein thiols, difference between Total thiols and free thiols was used (Table 1, Figure 1).

Determination of NO (Nitric Oxide)

1% Sulfanilamide Solution in 5% o-phosphoric acid and 0.1 % N-(1-Naphthyl)ethylene diamine dihydrochloride Solution was allowed to equilibrate to room temperature .50µl of stand serum sample was added. To this then, 50µl of the 1% Sulfanilamide Solution was added. 50µl of 0.1 % NED Solution was added. A purple/magenta color of Azo-compound will begin to form immediately. The absorbance was taken at 520 nm [8].

Estimation of MDA (Malondialdehyde)

Lipid peroxides were estimated by measurement of Thio barbituric acid reactive substances (TBARS) in plasma by the method of Brown and Kelly [9]. The pink chromogen produced by the reaction of Thiobarbituric acid with TBARS, a secondary product of lipid peroxidation was measured at 532 nm .Results was estimated as nmole/mL for serum.

Estimation of Advanced oxidation protein products (AOPP)

Serum AOPP was measured according to the method of Witko- Sarsat et al. [10,11]. In test wells, 200 µL of serum samples, diluted 1/20 in PBS, and was placed on a 96-well microtitre plate. 20 µL of acetic acid was added and 10 µL of 1.16 M potassium iodide was added to each well and the absorbance of the reaction mixture was immediately read at 340 nm on the microplate reader. AOPP concentrations were estimated using a standard curve of chloramine-T and expressed as uM.

C-Reactive Protein

Serum CRP was estimated using the commercially available kit (Transasia biomedical Co.). According to the manufacturer instructions, 30 µL of standards and samples with 500 µL of buffer were added to tubes. Optical density (O.D 1) of standards, controls and samples at 340nm was read. To this 50 µL of CRP antiserum was added; the solutions were mixed and incubated for 5 minutes at room temperature. Optical density (O.D 2) of standards, controls and samples at 340nm again measured. O.D. was calculated using (i.e. O.D 2- O.D 1), a standard curve was plotted and the concentration of controls and samples were measured.

Superoxide Anion

Superoxide anion was measured from PBMCs using the Nitro blue Tetrazolium salt to an insoluble blue formazan [12].

To 100 μL PBMC's (cell count =1×10⁶ cells), 100 μL of 1mg/mL of NBT solution was added. The cells were incubated overnight following incubation the tubes were centrifuged at 1500 rpm for 15 minutes and the supernatant was discarded. The pellet was with PBS to remove the excess of medium. The cells were then treated with 120 μL of 2N Potassium hydroxide and 140 μL of DMSO. The supernatant was measured at 650 nm.

Catalase activity

Serum Catalase activity was carried out by the method described by Abei [13] Catalase activity was measured spectrophotometerically at 240 nm. The reaction mixture containing 2.9 ml of phosphate buffer pH 7.4 with 30 mM H_2O_2 and 10 μL of serum sample. The reaction was measured for 3 minutes and the enzyme activity was express in U/μg of protein (Table 1, Figure 1).

Superoxide dismutase activity

Superoxide dismutase activity was carried out by the method described by Marklund and Marklund on based on auto-oxidation of pyrogallol in alkaline solution [14]. The reaction mixture containing 2 mL of Tris–cacodylate buffer pH 8.5 and 250 μL of sample was added and the reaction was started addition of 30 mM pyrogallol. The rate of auto-oxidation was measured as the incremental difference in A_{420} (ΔO/D) for 3minutes on UV spectrophotometer. A single unit of enzyme was expressed as 50% inhibition of Pyrogallol and enzyme activity was expressed in U/ ug of Protein (Table 1, Figure 1).

Glutathione S-transferase (GST) activity

Glutathione *S*-transferase (GST) activity was determined spectrophotometerically by the method of Habig *et al.* [15]. The reaction mixture (3 ml) contained 2.8 ml of 0.3 mM phosphate buffer (pH 6.5), 100μl of 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB). After pre-incubating the reaction mixture at 37°C for 5 min the reaction was started by the addition of 100μl serum and 100μl of glutathione as substrate. The absorbance was followed for 5 min at 340 nm. GST activity was expressed in nmole/min/ mL (Table 1, Figure 1).

Statistical Analysis

Each result was expressed as mean ± SEM. The statistical significance of the data was determined by one-way ANOVA test with Bonnferoni comparison. Statistical analysis was done using Graph Pad Prism 5 Software

Results and Discussion

Oxidative stress results from an imbalance between the generation of reactive oxygen and protective mechanisms. Free radicals, the main causes of oxidative stress, may react with variety of biomolecules including lipids, carbohydrates, proteins, nucleic acids and macromolecules of connective tissue. The oxidative stress is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases. Reduced glutathione (GSH) is the most prevalent non-protein thiol in animal cells. GSH levels have an impact on many immune functions, including activation of lymphocytes.

Table 1: Biochemical Parameters for Anti-Oxidants.

Parameters		Study Group			
		Control	GR-1	GR-2	GR-3
P-SH	Mean μM	315	691	669	679
	SEM (±)	24	75	73	119
	p Value	-	**	***	**
T-SH	Mean μM	359	739	706	714
	SEM (±)	27	85	74	122
	p Value	-	**	***	**
F-SH	Mean μM	45	48	37	35
	SEM (±)	7.9	12	5.1	6.1
	p Value	-	Ns	ns	Ns
SOD	Mean U/μg Protein	515.9	414.2	468.8	484.0
	SEM (±)	111.1	104.2	130.5	166.7
	p Value	-	Ns	ns	Ns
CAT	Mean U/μg Protein	175342	1217	900.3	590.0
	SEM (±)	39222	791.1	256.9	196.9
	p Value	-	**	***	*
GST	Mean U/μg Protein	1439	644.4	785.5	714.0
	SEM (±)	60.40	91.80	77.53	63.67
	p Value	-	***	***	***

Table 2: Biochemical Parameters For Pro-Oxidant.

Parameters		Study Group			
		Control	GR-1	GR-2	GR-3
NO	Mean μM	6.21	24.04	17.67	13.59
	SEM (±)	0.65	7.54	4.05	6.31
	p Value	-	*	ns	ns
TBARS	Mean nmol/mL	0.71	0.50	0.61	1.20
	SEM (±)	0.08	0.10	0.06	0.78
	p Value	-	Ns	ns	ns
AOPP	Mean μM	1048	890	942.6	988
	SEM (±)	119.7	207.8	155.4	253
	p Value	-	Ns	ns	ns
SOA	Mean OD Units	0.09	0.86	0.68	0.45
	SEM (±)	0.01	0.02	0.01	0.07
	p Value	-	***	***	***
CRP	Mean μM	5.18	5.47	4.32	6.96
	SEM (±)	1.60	4.45	2.11	4.25
	p Value	-	Ns	ns	ns

Consequently, it was postulated that GSH deficiency could lead to the progression of immune dysfunction, a hallmark of AIDS. GSH levels are depleted in plasma, epithelial lining fluid, peripheral blood mononuclear cells and monocytes in asymptomatic HIV-infected individuals and in AIDS patients. *In vitro* studies showing that low GSH levels both promote HIV expression and impair T cell function suggested a link between GSH depletion and HIV disease progression [16]. Systemic GSH deficiency has also been reported in symptom-free HIV seropositive individuals. Clinical studies have shown that GSH deficiency is correlated
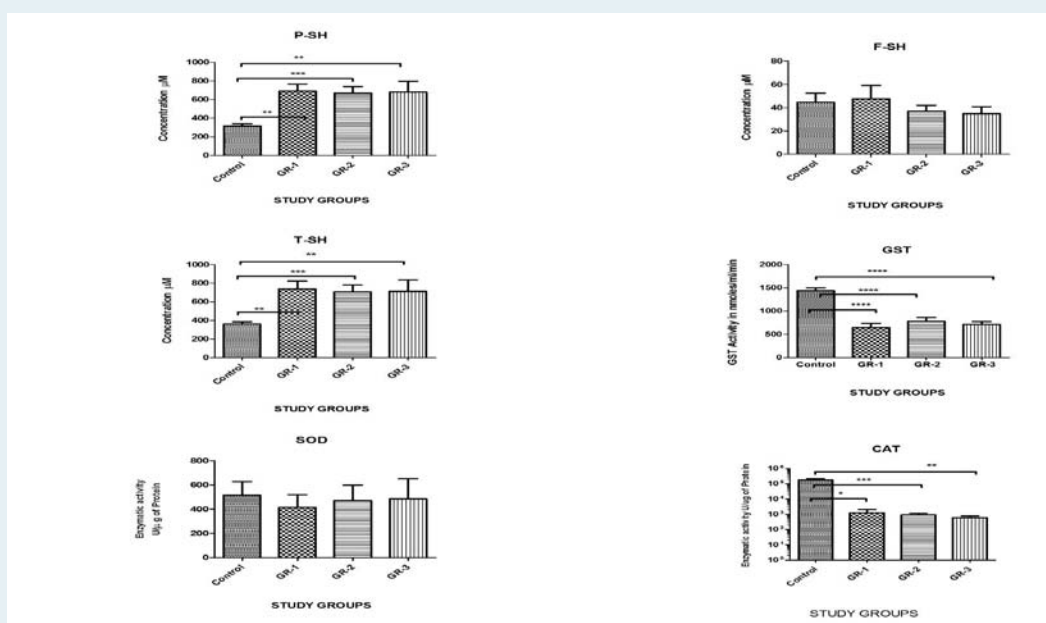
Figure 1: Activity of Antioxidants in the Study population. *p<0.01, ** p<0.001, *** p<0.0001.
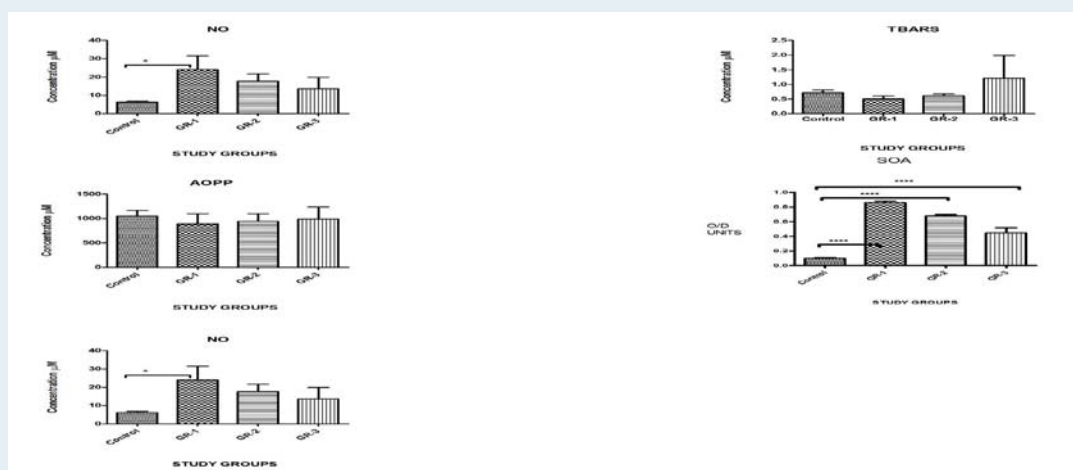

Figure 2: Activity of pro-oxidants in the study population. *p<0.01, ** p<0.001, *** p<0.0001.

with morbidity. It seems reasonable to conclude that a generally impaired antioxidant system is an obvious contributory factor that may contribute to these clinical findings, however, the precise importance of GSH deficiency presents a more complex scenario. Decreased GSH levels have been shown to activate NFκB, leading to a series of downstream signal transduction events that allow HIV expression. The long terminal repeat of HIV contains an NFκB site. *In vitro* studies have shown that NFκB binds to and activates genes controlled by the HIV LTR. NAC supplementation blocked HIV LTR gene expression, thereby confirming the importance of thiol status in HIV positive cells. In the present study, the protein thiols levels found to be increased significantly in HIV infected patients compared to normal control while the free thiol there is no significantly decreased in HIV

patients compared with normal controls and total thiol levels found to be elevated significantly in HIV/AIDS patients compared to normal control. The mean serum protein thiols levels in GR-1, GR-2 and GR-3 groups of HIV/AIDS patients were 679.1 μM, 689 μM and 679 μM respectively. These protein thiols values found to be increased when compared to control (mean 315 μM) [17]. The mean serum total thiols levels were found 359 μM, 739 μM and 714 μM in GR-1, GR-2 and GR-3 groups of HIV/AIDS infected patients, which were significantly increased to normal control (mean 359 μM). The mean serum free thiol levels were found 45 μM, 48 μM and 37 μM in GR-1, GR-2 and GR-3 groups of HIV/AIDS patients who were significantly high compared to normal control (mean 45 μM).

Decreased GSH, increased protein and total thiol levels may

be due to enhanced free radical generation in HIV/AIDS patients. These reduced thiol groups were oxidized by electron deficient free radicals, in the process of oxidation of –SH groups present over serum proteins occurs. Since –SH groups are the major antioxidants that contribute to the antioxidant pool of the body fluids, hence oxidation of such –SH groups can significantly contribute to the oxidative damage to biomolecules in HIV infected patients. Depletion of the CD4+ T-cell lymphocytes accompanies the etiology of HIV progression. Decreased GSH levels are known to be one contributory factor in the induction of apoptosis in CD4+ T lymphocytes. Superoxide dismutase is a group of metallo-enzymes that scavenges superoxide radicals and reduces their toxicity. It is an antioxidant that dismutase the O_2^- anion to form O_2 and H_2O_2. Animal cells contain two intracellular forms of SOD: the cytoplasmic or copper-zinc form (Cu-Zn SOD) and the mitochondrial or manganese form (Mn-SOD). This enzyme is the first line of defense against superoxide anion radicals.

In the present study, mean SOD levels were found to be 414.2 U/mg proteins, 468.8 U/mg proteins and 484.0 U/mg proteins in GR-1, GR-2 and GR-3 groups of HIV/AIDS infected patients respectively. The present study revealed a decrease in the level of SOD in serum of HIV/ AIDS patients than control group but this decrease was statistically insignificant [18,19]. Such decrease may be due to its detoxification of released ROS (superoxide anion). In HIV/AIDS infected patients, the autoxidation 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. The primary catalytic cellular defense that protects cells and tissues against potentially destructive reactions of superoxide radicals and their derivatives is the Cu/Zn-SOD. The co-relation between thiols and SOD was also stated in past studies indicating that auto-oxidation of thiols leads to formation of superoxide anion. As in the present study the total thiol level were increased which might leads to its auto-oxidation leading to more production of superoxide anion because of which SOD utilized in its detoxification leading decreased level of it. Catalase is an enzyme present in most of the aerobic cells, it protects them from oxidative stress by exerting a dual function; it catalyzes the decomposition of hydrogen peroxide to produce water and oxygen catalytic function or oxidation of H donors i.e. peroxidase function.

The mean catalase level were found to be 1217 U/mg proteins and 900.3 U/mg proteins in GR-1 and GR-2 group respectively while in GR-3 group 590.0 U/mg proteins. The catalase levels were found to be significantly decreased in GR-1 and GR-2 group while non-significantly increased in GR-3 group compared to normal control (mean 175342.1) [18,19]. The decrease in catalase activities in HIV infected patient's accounts for the production of high reactive oxygen species suggested that increased oxidative stress might be attributed to the deficiency of antioxidant defense system. This deficiency in HIV infected patients may be due to increased utilization of catalase. The increased activity found in GR-3group is may be due to as production of ROS or free radicals is less and the CD4 count is not less as compared to GR-1 and

GR-2 group. GSTs are a family of Phase II detoxification enzymes that have co-evolved with GSH and are abundant throughout most life forms. GSTs catalyze the conjugation of GSH to a wide variety of endogenous and exogenous electrophilic compounds. Human GSTs are divided into two distinct super family members; the membrane bound microsomal and cytosolic family members. Microsomal GSTs play a key role in the endogenous metabolism of leukotrienes and prostaglandins. Cytosolic GSTs are divided into six classes: a, μ, ω, π, θ, ζ.

In the present study, GST level was found to be decreased significantly in HIV/AIDS infected patients. The mean serum GST levels were 644.4 nmole/min/ml, 785.5 nmole/min/ml and 714.0 nmole/min/ml in GR-1, GR-2 and GR-3 group respectively were low compared to normal control (mean 1439 nmole/min/ml) [18,19]. The decreased activity might be due low levels of GSH and as GSH is substrate for the conjugation reactions which are catalyzed by GST. Nitric oxide is an important molecule to study the oxidative stress markers in the viral infections. The nitric oxide serves as a pro-oxidant molecule. The means serum nitric oxide levels in GR-1, GR-2 and GR-3 groups of HIV/AIDS patients were 24.04 μM, 17.67 μM and 13.59 μM respectively. These levels were significantly increased while compared to normal controls 6.213 μM.

Also the production of Superoxide Anion by the PBMC's was greatly enhanced in the HIV/AIDS patients with respect to the Control group. The Superoxide Anion levels increased significantly, 0.86 O/D units, 0.68 O/D units and 0.45 O/D units in GR-1, GR-2, and GR-3 O/D units when compared with normal control (0.099 O/D units). At higher concentrations, Nitric Oxide combines with Superoxide anion to from peroxynitrite ($ONOO^-$). A 10-fold increase in superoxide and NO production will increase peroxy nitrite formation 100-fold [20]. The synthesis of a even a moderate flux of peroxy nitrite over prolonged periods of time will result in considerable oxidation and potential damage of host cellular constituents, leading to the dysfunction of significant cellular processes, interruption of cell signaling pathways, and the induction of cell death through both apoptosis and necrosis. The lipid peroxidation is major process, which involves the oxidative degradation of lipids. The TBARS are a by-product of lipid peroxidation. The mean serum TBARS levels in GR-1, GR-2 and GR-3 groups of HIV/AIDS patients were 0.50 μM, 0.61μM and 1.20 μM respectively. These levels showed no significant increase while compared to normal controls 0.71 μM.

The advance protein oxidation potential (AOPP) serves as a potential biomarker to assess the protein damage occurred due to the oxidative stress. AOPP are defined as dityrosine containing cross-linked protein products AOPP levels correlated with concentrations of dityrosine and advanced glycation end products-pentosidine as indices of oxidant-mediated protein damage. AOPP are considered as reliable markers to estimate the degree of oxidant-mediated protein damage. The mean serum AOPP levels in GR-1GR-2 and GR-3 groups of HIV/AIDS patients were 49.40 μM, 47.13 μM and 44.50 μM respectively. These levels showed significant decrease while compared to normal controls 52.40 μM. C-reactive protein (CRP) is a marker to study the HIV disease progression. The mean serum CRP levels in GR-

1, GR-2 and GR-3 groups of HIV/AIDS patients were 5.47, 4.32 and 6.96 mg/L respectively. These levels showed no significant increase while compared to normal controls 5.18 mg/L. CRP is an acute phase protein whose elevation indicates of inflammatory conditions. Previous studies have used CRP as a marker for prognosis of co-morbid conditions associated with HIV patients [21-23]. These results are evidently indicative of oxidative stress in the HIV sero-positive study population. However, apart from the HIV-host interactions, opportunistic infections also have considerable role to play in the pathogenesis and hence the prognosis of HIV disease. Very few studies from India have comprehensively reported the various facets of association of Oxidative stress in the prognosis of HIV/AIDS. More detailed investigations shall pave way to a greater understanding of the pathologies caused by the redox imbalance in HIV/AIDS patients in India. Also taking a clue from these studies, development of defined antioxidant supplement regime in HIV becomes imperative. With the growing number of problems associated with the current treatment protocols it is only rational that we critically consider the alternatives.

Acknowledgement

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