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Enzymatic Oxidative Stress Indicators and Oxidative Stress Index in Patients of Leprosy

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Abstract

Introduction: Leprosy is a chronic granulomatous disease caused by Mycobacterium leprae. Oxidative stress caused by Reactive oxygen species (ROS) plays a crucial role in the pathogenesisis of leprosy.

Objective: To measure the enzymatic oxidative stress indicators and oxidative stress index in patients of leprosy and compare them with healthy age & sex matched controls.

Materials and Methods: In this prospective study 30 untreated leprosy patients were included in the study and matched with 60 healthy controls. Biochemistry estimation was done with blood samples and MDA (lipid peroxidation), Nitric oxide (NO), (SOD) Superoxide dismutase (antioxidant enzyme), total oxidant status (TOS), total antioxidant status(TAS) and oxidative stress index (OSI) were estimated.

Results: Highly significant rise ($p<0.0001$) in serum MDA, NO, TOS and OSI was seen in leprosy patients when compared with controls with highly significant decline in SOD and TAS.

Conclusion: The study confirms the oxidative stress in leprosy and suggests antioxidant therapy may be used as an adjuvant in the treatment of leprosy along with MDT.

Key words: Lipid peroxidation; Mycobacterium leprae; oxidants; reactive oxygen species; superoxide dismutase

Introduction

Leprosy (Hansen's disease) is a chronic, granulomatous, mutilating, visible, ancient, stigmatizing disease caused by Mycobacterium leprae and clinically manifests as nodules, plaques, thickened dermis and peripheral nerves.[1]

Oxidative stress (OS) caused by Reactive oxygen species (ROS) plays a crucial role in the pathogenesisis of leprosy.[2] OS is defined as excessive generation of ROS.[3] Normally the antioxidant defense system is responsible for balancing the production of ROS but in leprosy the production of ROS exceeds the normal limits resulting in OS causing further insult to cells, tissue and biomolecules.[3] Experimental studies support the possible relation between ROS and leprosy.[4]

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The 'antioxidant defense system' comprises of enzymatic scavangers of ROS, like SOD (Superoxide dismutase), catalase and glutathione peroxidise and nonenzymatic antioxidants like vitamin E, C and glutathione.[3] This dynamic defense system removes the aggressive oxygen species and keeps the concentration of ROS at normal levels.[3]

ROS have only transient existence and cannot be measured directly in vitro; hence the end products of OS are used as laboratory markers.[5] Among the

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biomarkers of OS, Lipid peroxidation (LPO) and Protein carbonylation are the most frequently used reliable laboratory parameters.[5] Measurement of serum antioxidants is another way to investigate OS.[6] SOD catalyzes the dismutation of superoxide ion into oxygen and hydrogen peroxide and is considered most reliable to determine enzymatic OS.[6] The present study was aimed to analyze the relationship between OS indices, and enzymatic OS indicators in leprosy cases and to find out whether these changes have a significant association with bacterial load and type of leprosy.

Recruitment of patients and controls:

In this prospective study, conducted in a tertiary care centre of North India, newly diagnosed adult leprosy patients registered in leprosy clinic from July 2014 to December 2015 were included based on below mentioned inclusion and exclusion criterion. The study was approved by the Institutional ethical committee and is registered in CTRI (CTRI/2017/08/009369). Newly diagnosed leprosy patients before the start of anti-leprosy treatment, were selected as cases. The patients were categorized according to the WHO classification based on clinical, histological and bacteriological criteria.[8] Clinical details included the number and distribution of lesions, pattern of nerve involvement, and complications including reactions, neuritis and deformities. The patients who were in reactions, taking any form of antioxidant/multivitamin supplementation, giving history of any other infectious diseases or other major systemic illnesses were excluded from the study. The Controls were age and sex matched and were apparently healthy individuals on general physical examination and without any past history of any chronic disease or leprosy. Above mentioned exclusion criteria for cases were also applied for controls. Persons with history of any addiction like smoking or alcoholic habits and other chronic skin or systemic diseases like diabetes mellitus, cancer or hypertension were also excluded from the study.

After initial screening according to the inclusion and exclusion criteria of all the patients, 60 were deemed fit for the inclusion in the study. Informed and written consent were taken from the patients for inclusion in the study. Out of 60, only 30 patients gave the consent for inclusion in the study. Blood sample was collected from 60 age and sex matched healthy controls and 30 leprosy Patients constituted the study groups.

Materials and Methods

Five ml blood was drawn from median cubital vein of the patients into plain tubes. To separate the serum from the plasma sample, it was centrifuged at 5000 × *g* for 5 min at room temperature. All serum samples were stored at -20°C until time of processing.

Lipid peroxidation was measured by the quantification of MDA (Malondialdehyde) in blood samples of patients using the thiobarbituric acid-reactive substances (TBARS) assay.[8] NO (Nitric oxide) was evaluated based on the principle that nitrate (NO3-) present in the serum samples is reduced to nitrite using nitrate reductase and the method is known as Griess Method.[9] Measurement of total oxidant status (TOS) was based on principle that oxidant present in the sample oxidizes the ferrous ion-o-dianisidine complex to ferric ions. The oxidation reaction is enhanced by glycerol molecules, which are enormously present in the reaction medium. During this oxidation reaction, the ferric ions make a coloured complex with xylenol orange in an acidic medium and the intensity of this colour is measured spectrophotometrically. This is directly related to the total amount of oxidant molecule present in the sample.[10] Superoxide Dismutase (SOD) was measured with help of Caymans superoxide dismutase assay kit, (item number 706002 from Caymen chemical company· 1180 East Ellsworth Road· Ann Arbor, Michigan 48108· USA). Total Antioxidant status (TAS) was measured with help of Caymans antioxidant assay kit, (item number 709001 from Caymen chemical company · 1180 East Ellsworth Road· Ann Arbor, Michigan 48108· USA).

The oxidative stress index (OSI) was calculated from a percent ratio of total oxidant level to the total anti-oxidant (TAS) level as explained elsewhere.[22]

OSI=[TOS (µmol H_2O_2 equivalent/L) / TAS (µmol Trolox equivalent/L) X100

Statistical analysis:

Results were expressed as mean±SD and these were evaluated using student paired and unpaired t test and z test. P value was considered highly significant if <0.0001 and significant if <0.05.

Results

The final study comprised of 30 patients and 60 healthy controls. Table 1 lists the general demographic and clinical characteristics of case group. Controls were age and sex matched and hence not statistically different from study patients (Table 1).

Status of enzymatic oxidative stress indicators between cases and controls:

It was observed that Malondialdehyde (MDA) in cases

was 0.89 (±0.32) µmol and in controls was 0.42 (±0.12) µmol This difference was highly significant. P <0.0001. On estimation of Nitric oxide (NO), in cases it was 160.92 (± 9.93) and mmol and in controls 47.26 (±10.15) mmol. This difference was also highly significant. P <0. 0001. The level of Superoxide dismutase (SOD) was significantly less in cases 2.43 (±1.82) µmol compared to controls 5.21(±0.34) µmol. (Table 2).

Status of oxidative stress indices in cases and controls: Total oxidant status was 34.90±15.55 mmol in controls and increased in cases 150.06±44.43 mmol. This difference was highly significant, p value was <0.0001. On the contrary, total antioxidant status was 1.09±0.12 µmol in controls and got decreased in cases, 0.39±0.26 µmol. This difference was also highly significant, p value was <0.0001. On calculating the oxidative stress index there was highly significant increase in cases, 73.39±29.98 mmol as compared with controls, 3.15±1.37 mmol (Table 3).

Baseline comparison of oxidative stress parameters with bacterial load and type of leprosy.
The total oxidant status (TOS), total antioxidant status (TAS), oxidative stress index (OSI) and Malondialdehyde (MDA) levels were decreased in Bacteriological index = 0 in comparison to Bacteriological index ≥ 1However, this was not statically significant. The Nitric oxide (NO) and Superoxide dismutase (SOD) were increased in Bacteriological index = 0 in comparison with Bacteriological index ≥ 1, And this increase was also not significant.

The total antioxidant status (TAS) and oxidative stress index (OSI) and Malondialdehyde (MDA) levels were decreased in Paucibacillary in comparison to Multibacillary with no statistical significance.

The total oxidant status (TOS), Nitric oxide (NO) and Superoxide dismutase (SOD) were increased in Paucibacillary in comparison with Multibacillary with no significant difference (Table 4).

Table 1: Demographic and clinical characteristics of case group.

Variables	Cases (n = 30)
Gender= n (%)	
Female	10(33%)
Male	20 (67%)
Age (%)	
18 – 30 years	15(50%)
30 – 50 years	11(37%)
50 – 70 years	4(13%)
PB/MB (%)	
PB	14(47%)
MB	16(53%)
Bacteriological index (%)	
BI = 0	17(57%)
BI ≥ 1	13(43%)
Morphological index% (n = 13)	
MI = 0	2(15%)
MI ≥ 1	11(85%)
Nerve involvement (%)	
N ≤ 1	16(53%)
N ≥ 2	14(47%)

Table 2: Status of enzymatic oxidative stress indicators between cases and controls (Z test).

Indicator	Cases n= 30 mean±SD	Controls n=60 mean±SD	t-test value	P value
Malondialdehyde (MDA µmol)	0.89 ± 0.32	0.42 ± 0.12	10.08	<0.0001
Nitric oxide (NO mmol)	160.92 ± 9.93	47.26 ± 10.15	50.43	<0.0001
Superoxide dismutase (SOD µmol)	2.43 ± 1.82	5.21 ± 0.34	11.49	<0.0001

SD:standard deviation

Table 3: Oxidative stress indicators of leprosy.

Category	Cases N=28	Controls N=40	t-test value	P value
Total Oxidant status (TOS mmol)	150.06 ± 44.43	34.90 ± 15.55	18.06	<0.0001
Total antioxidant status (TAS µmol)	0.39 ± 0.26	1.09 ± 0.12	13.68	<0.0001
Oxidative stress index (OSI mmol)	73.39 ± 29.98	3.15 ± 1.37	18.21	<0.0001

Table 4: Comparison of oxidative stress parameters with bacterial load and type of leprosy (One way ANOVA test).

Variables	Gender			AGE (Years)			Type of leprosy			BACTERIOLOGICAL INDEX		
	MALE (20)	FEMALE (10)	CONTROLS (60)	15-30 (15)	>30 (15)	CONTROLS (60)	PB (14)	MB (16)	CONTROLS (60)	BI = 0(17)	BI≥1(13)	CONTROLS (60)
MDA (µmol)	0.91± 0.36**	0.83± 0.21**	0.42± 0.12	0.820± 0.20**	1.00± 0.464**	0.42± 0.12	0.85 ± 0.29**	0.97 ± 0.40**	0.42± 0.12	0.87± 0.34**	0.91 ± 0.34**	0.42± 0.12
NO (mmol)	158.76± 0.41	163.50± 8.83	47.26± 10.15	158.27± 8.74	159.78± 8.78	47.26± 10.15	162.70± 11.30	157.90± 7.45	47.26± 10.15	160.80± 10.80	158.24 ± 6.65	47.26± 10.15
SOD (µmol)	2.03 ± 0.41**	3.23 ± 3.37**,#	5.21± 0.34	2.09 ± 0.42**	2.46 ± 2.22**	5.21± 0.34	2.78± 2.45**	1.97± 0.41**	5.21± 0.34	2.53± 2.00**	1.92 ± 0.43**	5.21± 0.34
TAS (µmol)	0.44± 0.32**	0.34± 0.27**	1.09± 0.12	0.367± 0.28**	0..48 ± 0.33**	1.09± 0.12	0.34 ± 0.18**	0.47 ± 0.37**	1.09± 0.12	0.38 ± 0.27**	0.48 ±0.0.37**	1.09± 0.12
TOS (mmol)	161.71± 48.04**	149.26± 42.04**	34.90± 15.55	155.25± 40.07**	166.58± 52.93**	34.90± 15.55	162.93± 45.67**	157.97± 48.04**	34.90± 15.55	158.67± 46.92**	159.97± 47.75**	34.90± 15.55**
OSI (mmol)	79.32 ± 85.56**	57.43± 46.73**	3.15± .37	71.04 ± 89.04**	79.25± 69.15**	3.15± .37	66.81± 60.71**	79.83 ± 89.47**	3.15± .37	60.58± 58.68**	94.39 ± 99.50**	3.15± .37**

highly significant with controls =**, significant with controls = *, significant = #

Discussion

The present study is a prospective study which includes 60 cases of healthy controls and 30 cases of leprosy patients. In modern era, interest has grown in investigating the role played by reactive oxygen species or oxidative stress in leprosy leprosy by investigating one or more of the oxidant markers like Malondialdehyde (MDA) and antioxidant markers, including SOD, catalase, vitamin E, vitamin C.[11-14]

Lipid peroxidation is involved in pathogenesis of many disease processes when the imbalance occurs between production of reactive oxygen species and protective antioxidant system. Malondialdehyde (MDA) is a marker of lipid peroxidation.[15] In the present study, MDA was significantly high in serum of leprosy patients (p <0.0001) in comparison to healthy controls. Agnihotri N, et al (1995) noticed increased levels of lipid peroxidation levels in mice infected with *M. leprae*.[4] Bhadwat VR et al in their study showed increased Malondialdehyde levels in leprosy patients and more so in lepromatous leprosy.[16]

In the present study, the activity of serum enzymatic antioxidant superoxide dismutase (SOD) was highly significantly low in leprosy cases as compared to control group (p <0.0001). Previous studies have also reported significant reduction of activity of serum SOD in untreated leprosy patients.[19,20] Regarding nitric oxide production, we observed that their levels were significantly higher in leprosy patients as compared to healthy controls (p<0.0001). The reason behind it is that Microbial killing of lepra bacilli results in production of free radicals like nitric oxide.[21] These findings are similar to the study done by Schalcher TR et al who also reported increased production of nitric oxide levels in leprosy patients as compared to healthy controls and no change in nitric oxide levels were seen after treatment with antileprosy drugs[11,14] It seems that the disease process itself appears to be responsible for the detected nitric oxide increase in the body irrespective of the antileprosytreatment.[14]

Studies have tried to quantify oxidative stress as per evaluating levels of various enzymes and carbonyl proteins. Some of the research analyst reported changes of these markers like Superoxide Dismutase (SOD), Glutathione peroxidase (GPx), Malondialdehyde (MDA), Nitric Oxide (NO), and Catalase.[1,12] But these studies didn't measure the total oxidative stress unlike our present study. Oxidative stress can be measured by calculating the ratio of total oxidant status (TOS) and total antioxidant status (TAS).[10,22] In the present study there was a highly significant increase in the total oxidant status in leprosy patients in comparison to healthy controls (P<0.0001) and there was a highly significant decrease in mean total antioxidant status in leprosy in comparison to healthy controls (P<0.0001). This supports the theory that generation of free radicals are involved in the pathogenesis of leprosy.[1,13]

When we measured oxidative stress index(OSI) by ratio of TOS/TAS, Highly Significant increase in the OSI was observed in patients of leprosy as compared to healthy controls (p <0.0001). Bhadwat VR et al reported increased oxidative stress by quantifying ratio of (MDA/SOD) in LL leprosy in comparison to TT leprosy.[16] Abdel-Hafez HZ et al also reported similar findings.[18] Jyothi P et al reported significant elevation in oxidative stress by measuring the ratio of (MDA/SOD) in MB leprosy.[17] Hence this study confirms the presence of oxidative stress in leprosy and intervention with antioxidant supplementation may improve the

total oxidant capacity of the patient by reducing the production of reactive oxygen species(ROS) which in turn improves the oxidative stress in leprosy.

Conclusion

Our study confirmed the oxidative stress in leprosy with highly significant increase in oxidative stress index in leprosy patients. The enzymatic antioxidant superoxide dismutase (SOD) was reduced in leprosy patients indicating enzymatic stress in leprosy. The drawback in our study was less number of patients and that we could not measure the nonenzymatic antioxidants in our patients. Further studies need to be done on a larger scale with estimation of enzymatic, non-enzymatic antioxidants and total oxidative stress in leprosy patients. These findings support a hypothesis that any enzymatic supplementation might have an attractive approach in combating oxidative stress mediated insult during the chronic course of the disease process of leprosy.

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