CIRCADIAN PERIODICITY OF PLASMA LIPID PEROXIDES AND ANTI-OXIDANT ENZYMES IN PULMONARY TUBERCULOSIS

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ABSTRACT

The circadian periodicity of plasma lipid peroxide levels and activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were studied in 50 clinically, bacteriologically and radiologically proven fresh cases of pulmonary tuberculosis (age: 21-45 years) and 60 age-matched healthy volunteers with diurnal activity from 06:00 to about 22:00 and nocturnal rest. A marked circadian variation in plasma lipid peroxide level was recorded in healthy subjects and pulmonary tuberculosis patients with significant amplitude and acrophase around 16:21 and 17:12 respectively. The acrophase tended to be delayed in tuberculosis patients. Furthermore, a statistically significant circadian rhythm was found in SOD, CAT and GPx activities in normal volunteers and pulmonary tuberculosis patients. SOD and CAT enzyme activity was noted to be maximum at 06:00 and minimum at 00:00 in tuberculosis patients. The circadian acrophase for GPx activity was recorded at 16:15 in normals and around 22:45 in patients. Moreover, the activity was found to be decreased at all sampling hours during 24-hours sleep-awake period in patients in comparison to healthy counterparts. The MESOR and circadian amplitude also decreased markedly. The decreased activity of measured antioxidant enzymes in pulmonary tuberculosis patients could probably be associated with oxidative stress and/or decreased anti-oxidant defensive mechanism in such patients.

INTRODUCTION

Tuberculosis has emerged as the leading cause of death from a single infectious agent in the world. India has nearly 30% of the global burden of tuberculosis. With an estimated incidence or new smear positive tuberculosis of 85 per 10,000 populations, there are nearly 1 million new smear-positive cases of tuberculosis a year and about 2 million total new cases.

Oxidative stress associated with inflammation has been implicated in the development of lung dysfunction in patients with tuberculosis. Plit *et al.* (1) have reported elevated circulating lipid peroxides and decreased plasma concentration of vitamin E suggesting a high level oxidative stress in patients with pulmonary tuberculosis. A circadian rhythm has been well established for most laboratory variables in western population (2-6). We have reported marked circadian variation in different blood and urinary variables in

healthy Indians (7-12). Little is known, however, about such rhythms in circulating lipid peroxides and antioxidant enzymes under tropical conditions. To our knowledge, the circadian nature and status of lipid peroxides and antioxidant defence system has not yet been reported in pulmonary tuberculosis. The present study aims to fill this gap by providing oxidant and antioxidant enzymatic circadian profile in pulmonary tuberculosis in an attempt to understand the mechanism of oxidative stress and their involvement in such pathological conditions.

MATERIALS AND METHODS

Fifty clinically, bacteriologically and radiologically proven fresh cases of pulmonary tuberculosis (group A; age: 21-45 years) and 60 clinically healthy volunteers of either sex (group B; age: 21-40 years) were included in this study. Only minimal and moderately advanced patients of pulmonary tuberculosis were included in the present study (13). All participants were synchronized for one week with diurnal activity from about 6:00 to about 22:00 hrs. and nocturnal rest. All subjects took their usual (although not identical) meals three times daily; breakfast around 8:30 a.m., lunch around 13:30 p.m. and dinner around 20:30 p.m., without any change in their usual fluid intake. The

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burden of environmental temperature and pollution, if any, was common to all participants. Prior to the blood sample collection, the participants refrain from taking any drugs/preparation that would affect or alter the oxidative stress, its defensive mechanism, level and rhythm. Six millilitres of blood was collected from each subject at fixed time points for one complete 24 hour cycle, at 06:00, 12:00, 18:00 and 00:00 hrs. in plain and sterile vials containing heparin as anticoagulant. The plasma was separated and analyzed for lipid peroxides in terms of malondialdehyde (MDA) (14). The haemolysate was prepared from the red cells and used for the measurement of the activities of enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (15,16,17).

Data were evaluated by conventional statistical analyses and by the single- and population-mean cosinor procedures (18,19). Accordingly, the MESOR (a rhythm-adjusted mean, usually more precise and more accurate than the arithmetic mean), the circadian amplitude (a measure of half the extent of predictable change within a 24-hour cycle; the double amplitude being a measure of total extent of change within a day

predictable by the curve fitted); and the circadian acrophase (a measure of the timing of overall high values of the fitted 24-hour cosine function) were estimated. Rhythm detection was assessed by the zero amplitude (no rhythm) test at the 5% probability level.

RESULTS AND OBSERVATIONS

Mean plasma MDA concentration, found to be 2.83 nmole/ml plasma at 18:00 hrs., decreased markedly during the rest of the period, reaching a minimum at 06:00 in healthy volunteers. These variations at different collection hours during a 24-hour cycle were significant ($p < 0.001$) with acrophase around 16:21. Cosinor rhythmometry in terms of MESOR (2.07 ± 0.04), amplitude (0.77 mole/ml plasma; 95% CI: 0.58 to 0.89) and acrophase (-258° from local midnight, with $360^\circ = 24$ hours; 95% CI = -241° to -265°) confirmed a marked circadian rhythm in plasma MDA level in pulmonary tuberculosis patients with acrophase around 17:12 ($F=69.22$; $p < 0.001$). By comparison to healthy controls, the tubercular patients had a delayed acrophase by nearly 13° or 50 minutes (Table-1, Fig. 1, 2).

Table 1: One-way ANOVA and single analysis of plasma MDA (n mole/ml plasma) level in age-matched healthy controls and pulmonary tubercular patients at different collection hours of 24-hour light-dark period

One-Way ANOVA

Source of variation	Normals		Patients	
	Among Times	Among Subjects	Among Times	Among Patients
Degree of freedom (DF)	03	59	03	49
Sum of Squares (SS)	32.79	45.39	35.98	13.32
Mean Squares (MS)	10.93	0.76	11.99	0.45
F	47.06	3.31	4.37	2.08
P	<0.001	<0.05	<0.001	>0.05

At the collection hours of 06:00, 12:00, 18:00 and 00:00; the mean plasma MDA levels were 1.91, 2.40, 2.83, 1.98 in healthy participants and 1.37, 2.17, 2.87 and 1.86 respectively in pulmonary tubercular patients.

Single Cosinor Analysis

Variable	No.	MESOR $\pm$ SE	Double Amplitude	Acrophase		F (nDF)	p value
				Degrees	Time		
Normals	60	2.28 $\pm$ 0.03	1.00	-245.37	16:21	67.09 (2,60)	<0.001
Patients	50	2.07 $\pm$ 0.04	1.54	-258.15	17:12	69.22 (2,50)	<0.001

M = MESOR (midline-estimating statistic of rhythm): 24-hour rhythm-adjusted mean;

A = amplitude: one-half of extent of change predictable by fit of single cosine curve;

F = acrophase: measure of the timing of overall high values recurring in each cycle (expressed in [negative] degrees, with $360^\circ = 24$ h; $0^\circ = 00.00$);

SE = Standard error

Fig. 1: Circadian rhythm of plasma lipid peroxides and anti-oxidant enzymes in healthy volunteers and pulmonary tuberculosis patients.

Fig. 2: Partial circadian acrophase chart in healthy volunteers and pulmonary tuberculosis patients

Cosinor analysis confirmed a marked circadian variation in SOD activity in healthy Indians with significant amplitude and acrophase at 07:22 ($F=143.95$; $p<0.001$; Table-2). SOD activity was calculated to be maximum at 06:00 and minimum at 00:00 in pulmonary tubercular patients. Cosinor

rhythmometry further, confirmed a marked circadian variation of SOD activity in tubercular patients with acrophase around 07:51 ($F=1.64$; $p>0.05$). However, MESOR and amplitude decreased significantly in patients in comparison to healthy controls exhibiting an overall decline of SOD activity at all collection hours of 24-hour cycle (Fig. 1,2).

Table 2: One-way ANOVA and single analysis of plasma SOD (Units/ml RBCs) activity in healthy volunteers and pulmonary tubercular patients at different collection hours of 24-hour cycle

One-Way ANOVA

Source of variation	Normals		Patients	
	Among Times	Among Subjects	Among Times	Among Patients
Degree of freedom (DF)	03	59	03	49
Sum of Squares (SS)	246.46	358.30	14.31	24.36
Mean Squares (MS)	82.15	6.07	4.77	1.74
F	54.03	3.99	4.96	1.08
P	<0.001	<0.05	<0.05	>0.05

At the sampling times of 06:00, 12:00, 18:00 and 00:00; the mean plasma SOD activity were 21.32, 20.43, 18.64 and 19.42 in age-matched healthy subjects and 16.66, 15.71, 15.91 and 15.31 respectively in pulmonary tuberculosis patients.

Single Cosinor Analysis

Variable	No.	MESOR $\pm$ SE	Double Amplitude	Acrophase		F (nDF)	p value
				Degrees	Time		
Normals	60	19.95 $\pm$ 0.05	2.86	-110.70	07:22	143.95 (2,60)	<0.001
Patients	50	15.90 $\pm$ 0.16	0.84	-117.95	07:51	1.64 (2,50)	>0.05

M = MESOR (midline-estimating statistic of rhythm): 24-hour rhythm-adjusted mean;

A = amplitude: one-half of extent of change predictable by fit of single cosine curve;

F = acrophase: measure of the timing of overall high values recurring in each cycle (expressed in [negative] degrees, with $360^\circ = 24$ h; $0^\circ = 00.00$);

SE = Standard error

Mean CAT activity was found to be 15.81 (units/ml RBCs) at 06:00, which decreased gradually to a minimum at 18:00 in healthy volunteers. Population-mean-cosinor analysis in terms of MESOR, amplitude and acrophase confirmed a marked circadian rhythm in CAT activity in healthy volunteers with significant amplitude and acrophase at 08:04 ($F=172.96$; $p<0.001$; Table-3). Maximum CAT activity was calculated to be 12.75 (units/ml RBCs) at 06:00 in pulmonary tuberculosis patients which declined regularly throughout the 24-hour cycle reaching a minimum at 00:00 with a mean of 10.47 (units/ml RBCs). These variations were significant at different sampling hours by one-way ANOVA ($p<0.01$) and by cosinor rhythmometry, confirming thereby the presence of a statistically significant circadian rhythm in CAT

activity in tubercular patients ($p<0.05$) with an acrophase around 09:34 (Table-3, Fig. 1,2).

Mean GPx activity was observed to be maximum at 18:00 and minimum at 00:00 in healthy controls. ANOVA showed significant variations in GPx activity at different collection hours which was further confirmed by cosinor analysis with significant amplitude and acrophase at 16:15 ($F=93.52$; $p<0.001$; Table-4). However, circadian rhythm was noticed to be deranged in pulmonary tuberculosis patients with maximum activity at 00:00 and minimum at 12:00. Cosinor rhythmometry in terms of MESOR (3.24 ± 0.04 nmole NADH oxidized/min/mg protein), amplitude (0.26; 95% CI: 0.08 to 0.44) and acrophase (-341° from local midnight; with $360^\circ \equiv 24$ hours; 95% CI: -37° to -287°) further confirmed a regular and consistent rhythm in GPx activity of

Table 3: One-way ANOVA and single analysis of plasma Catalase (Units/ml RBCs) activity in age-matched healthy controls and pulmonary tubercular patients

One-Way ANOVA				
Source of variation	Normals		Patients	
	Among Times	Among Subjects	Among Times	Among Patients
Degree of freedom (DF)	03	59	03	49
Sum of Squares (SS)	201.02	37.19	43.29	18.05
Mean Squares (MS)	67.00	0.63	144.43	1.28
F	154.96	1.45	24.24	2.16
P	<0.001	>0.05	<0.01	>0.05

At the sampling times of 06:00, 12:00, 18:00 and 00:00; the mean plasma Catalase activity was noted to be 15.81, 15.05, 13.63 and 13.73 in healthy volunteers and 12.75, 12.18, 11.49 and 10.47 respectively in pulmonary tuberculosis patients.

Single Cosinor Analysis							
Variable	No.	MESOR±SE	Double Amplitude	Acrophase		F (nDF)	p value
				Degrees	Time		
Normals	60	14.55±0.04	2.54	-121.21	08:04	172.96 (2,60)	<0.001
Patients	50	11.72±0.15	2.12	-143.73	09:34	12.33 (2,50)	>0.05

M = MESOR (midline-estimating statistic of rhythm): 24-hour rhythm-adjusted mean;

A = amplitude: one-half of extent of change predictable by fit of single cosine curve;

F = acrophase: measure of the timing of overall high values recurring in each cycle (expressed in [negative] degrees, with 360° = 24 h; 0° = 00.00);

SE = Standard error

pulmonary tuberculosis patients with an amplitude significantly different from zero and acrophase around 22:45 (F=7.52; p<0.05; Table-4). Amplitude decreased markedly in patients with decreased MESOR and delayed acrophase by nearly 100° or 6 hours in comparison to healthy controls (Fig. 1,2).

DISCUSSION

A marked circadian variation in plasma MDA level was recorded in healthy Indians and pulmonary tuberculosis patients with significant amplitude and acrophase around 16:21 and 17:12 respectively. The circadian acrophase of plasma MDA levels occurred around 50 minutes later in patients in comparison to healthy controls; however, there was no significant difference in the MESORS of the two groups. The circadian amplitude tends to be increased in pulmonary tuberculosis patients in comparison to the healthy volunteers. Plit *et al.* (1) have reported elevated levels of serum lipid peroxides in patients with active pulmonary tuberculosis along with decreased plasma concentrations of vitamin E. They have suggested a possible role for supplementation with Vit E in such conditions. However, plasma lipid peroxide levels in terms of MDA concentrations were normal in the

present study, though there was a shift in circadian acrophase exhibiting the involvement of oxidative stress in such situations. Moreover, only minimal and the moderately advanced group of patients were included in this study. The present observations regarding circadian nature of plasma MDA levels in healthy volunteers were in agreement with other reports (20-22). There are no reports regarding circadian variations of circulating lipid peroxides in pulmonary tuberculosis patients under tropical conditions.

A statistically significant circadian rhythm was recorded in SOD, CAT and GPx concentrations in clinically healthy subjects and pulmonary tuberculosis patients. SOD activity was found to be maximum at 06:00 hrs. and minimum at 00:00 hrs. in patients. Moreover, the activity was noticed to be decreased at all sampling hours during a 24-hour sleep-awake period in patients in comparison to their healthy counterparts. The MESOR and circadian amplitude also decreased markedly in pulmonary tuberculosis patients suggesting decreased SOD concentrations. The circadian acrophase occurred 30 minutes later in patients as compared to healthy volunteers. Similarly, CAT activity was also noticed to be reduced at all collection hours

Table 4: One-way ANOVA and single analysis of glutathione peroxidase (n mole NADH oxidized/min/mg protein) in healthy subjects and pulmonary tuberculosis patients

One-Way ANOVA

Source of variation	Normals		Patients	
	Among Times	Among Subjects	Among Times	Among Patients
Degree of freedom (DF)	03	59	03	49
Sum of Squares (SS)	73.46	7.72	2.15	3.34
Mean Squares (MS)	24.48	0.13	0.71	0.23
F	104.78	0.56	4.38	1.45
P	<0.001	>0.05	<0.05	>0.05

At the sampling times of 06:00, 12:00, 18:00 and 00:00; the mean plasma GPx activity was found to be 3.67, 4.27, 4.99 and 3.62 in healthy subjects and 3.12, 3.02, 3.29 and 3.52 in patients respectively.

Single Cosinor Analysis

Variable	No.	MESOR $\pm$ SE	Double Amplitude	Acrophase		F (nDF)	p value
				Degrees	Time		
Normals	60	4.14 $\pm$ 0.03	1.46	-243.81	16:15	93.52 (2,60)	<0.001
Patients	50	3.24 $\pm$ 0.04	0.52	-341.43	22:45	7.52 (2,50)	<0.05

M = MESOR (midline-estimating statistic of rhythm): 24-hour rhythm-adjusted mean;

A = amplitude: one-half of extent of change predictable by fit of single cosine curve;

F = acrophase: measure of the timing of overall high values recurring in each cycle (expressed in [negative] degrees, with 360° = 24 h; 0° = 00.00);

SE = Standard error

in patients with decreased MESOR and circadian amplitude. The circadian acrophase occurred around 1 hour and 30 minutes later in patients. GPx activity further decreased at all collection hours in tuberculosis patients with maximum activity at 00:00 and minimum at 12:00. The MESOR and circadian amplitude decreased markedly. Moreover, there was maximum swing and altered rhythm in the activity of this important antioxidant enzyme where the circadian amplitude was found to occur about 6 hours and 30 minutes later in patients in comparison to healthy subjects. Copper and Zinc containing SOD, manganese containing SOD, CAT and GPx form the primary enzymatic defense against reactive oxygen metabolites. Such metabolites have been implicated in the damage brought about by ionizing radiation, as well as in the effects of several cytostatic compounds (23). The decreased concentration of measured antioxidant enzymes in pulmonary tuberculosis patients could probably be associated with oxidative stress and/or decreased antioxidant defense mechanism.

GPx activity, found to be decreased at all sampling hours in patients in comparison to healthy subjects, clearly exhibited an imbalance between oxidant and antioxidant defensive systems in the human body under such pathological situations. This probably could be the important factor for the etiopathogenesis

of the disease and needs further confirmation for better supplementation of transition metals and micronutrients along with antioxidant vitamins with known antitubercular regimen. Furthermore, supplementation of today's cocktail of tubercular drugs with antioxidant vitamins and nutrients should be advocated in the right combination and for the right length of time according to the biological clock for better therapeutic utilization and efficiency.

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