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Research Article

Circadian Rhythm of Plasma Lipid Peroxidation and Total Antioxidant Status in Patients with Alcoholic Hepatitis: A Comparative Cross-Sectional Study

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ABSTRACT

Background: Oxidative stress is central to the pathogenesis of alcoholic liver disease, and both the generation of reactive oxygen species and antioxidant capacity are under circadian control. Most clinical studies, however, have measured oxidative-stress markers at a single time of day. This study characterised the twenty-four-hour profile of plasma malondialdehyde (MDA), a marker of lipid peroxidation, and of total antioxidant status (TAS), an integrated measure of antioxidant capacity, in patients with alcoholic hepatitis and in healthy controls.

Methods: In a cross-sectional comparative study with repeated measures over twenty-four hours, thirty patients with alcoholic hepatitis and thirty age- and sex-matched healthy controls were studied. Venous blood was sampled at 06:00, 12:00, 18:00 and 00:00 h. Plasma MDA was estimated by the thiobarbituric-acid method and TAS by a standardised assay. Between-group differences at each time were tested by Student's *t*-test; circadian characteristics (MESOR, amplitude, acrophase) were derived by single-cosinor rhythmometry.

Results: A significant circadian rhythm of MDA was demonstrable in both groups. In patients the MDA MESOR was significantly higher (5.16 ± 0.71 versus 3.39 ± 0.38 nmol/ml; $p < 0.001$), the amplitude reduced (0.41 ± 0.12 versus 0.64 ± 0.15; $p = 0.002$) and the acrophase advanced from the late afternoon (≈16:21 h) towards midday (12.18 ± 1.5 versus 16.35 ± 1.2 h; $p < 0.001$). Plasma TAS was significantly lower in patients at every sampling time (all $p < 0.001$) and its diurnal variation was blunted.

Conclusion: Patients with alcoholic hepatitis show an elevated, phase-advanced and amplitude-attenuated rhythm of lipid peroxidation together with a persistently depressed total antioxidant status across the twenty-four-hour cycle, indicating that oxidative injury is both quantitatively increased and temporally dysregulated. As a cross-sectional study this work cannot establish causation; the findings are hypothesis-generating for the time-qualified interpretation of oxidative-stress markers and for future chronotherapeutic research.

Keywords: Alcoholic Hepatitis, Circadian Rhythm, Malondialdehyde, Lipid Peroxidation, Total Antioxidant Status, Cosinor Rhythmometry, Oxidative Stress.

INTRODUCTION

Alcoholic liver disease (ALD) remains a major preventable cause of chronic liver morbidity and mortality worldwide, spanning a continuum from steatosis through alcoholic hepatitis to fibrosis, cirrhosis and hepatocellular carcinoma [1,2]. Alcoholic hepatitis, the clinically florid inflammatory stage, is of particular importance because its severe form carries a high short-term mortality and because the pro-oxidant and pro-inflammatory processes that drive the disease are especially active at this stage [2,3].

Among the mechanisms proposed to explain alcohol-induced hepatocellular injury, oxidative stress has emerged as a unifying concept [3,4,5]. Ethanol metabolism, in particular through the inducible microsomal enzyme cytochrome P450 2E1, together with mitochondrial dysfunction and Kupffer-cell activation, increases the generation of reactive oxygen species (ROS) beyond the capacity of cellular antioxidant defences [4,5,6]. The excess reactive species peroxidise the polyunsaturated fatty acids of membrane phospholipids; the resulting lipid hydroperoxides decompose to reactive aldehydes, of which malondialdehyde (MDA) is the most widely measured [6,7]. Elevated circulating MDA has been reported consistently

across the spectrum of ALD and broadly parallels the intensity of hepatocellular injury [8,9,10,11].

Alongside markers of oxidative damage, the integrated antioxidant buffering capacity of plasma can be captured in a single measurement as the total antioxidant status (TAS), which reflects the combined contribution of the enzymatic and, predominantly, non-enzymatic antioxidants [12,13]. A reduced TAS in ALD would indicate a globally weakened defence against oxidative challenge [11].

A dimension of oxidative-stress biology that has attracted increasing attention is its temporal organisation. Virtually all cellular processes relevant to redox balance — mitochondrial respiration, ROS generation and the expression and activity of antioxidant systems — vary in an approximately twenty-four-hour (circadian) manner under the direction of the molecular clock, so that the oxidative burden experienced by a cell is not constant but oscillates over the day [14,15,16,17]. The rhythmic secretion of melatonin, itself a radical scavenger and inducer of antioxidant defence, provides a further link between the clock and redox biology [18]. A direct corollary follows for laboratory medicine: because a rhythmic analyte takes different values at different clock times, a single measurement provides only a partial snapshot of an individual's true oxidative-stress status, and the time of sampling must be taken into account [19,20]. Despite this, the great majority of clinical studies of oxidative stress in ALD have relied on single, and frequently unspecified, sampling times, and few have characterised the full twenty-four-hour profile of these markers, particularly in Indian populations studied under near-tropical conditions [11,21].

The present study was therefore undertaken to characterise and compare the twenty-four-hour profile of plasma MDA and of TAS in patients with alcoholic hepatitis and in healthy controls, and to quantify the circadian characteristics of MDA by cosinor rhythmometry. The specific questions were whether the mean level (MESOR), the amplitude and the timing (acrophase) of the MDA rhythm differ between patients and controls, and whether the integrated antioxidant capacity is reduced across the whole day.

MATERIALS AND METHODS

Study Design and Setting

A cross-sectional, comparative study with repeated measurements over a single twenty-four-hour period was conducted in the Department of Biochemistry, Index Medical College, Hospital & Research Centre, Indore, under Malwanchal University.

Participants

Thirty patients (aged 18–70 years) with a clinical and biochemical diagnosis of alcoholic hepatitis and thirty age- and sex-matched healthy volunteers were enrolled. The diagnosis of alcoholic hepatitis was based on a history of significant chronic alcohol consumption together with compatible clinical features and supportive laboratory findings. Inclusion criteria for patients were: age 18–70 years, a confirmed history of chronic alcohol consumption, clinical and biochemical evidence of alcoholic hepatitis, and willingness to comply with the twenty-four-hour sampling schedule. Exclusion criteria were: viral hepatitis, autoimmune liver disease, hepatocellular carcinoma, severe systemic illness, the use of antioxidant or hepatoprotective drugs in the preceding four weeks, and inability to comply with the sampling schedule. Healthy controls had no history of alcohol abuse, liver disease or other chronic illness.

Sample Collection

Venous blood samples were collected from each participant at four time points over twenty-four hours — 06:00, 12:00, 18:00 and 00:00 h — under standardised conditions. Plasma was separated and processed for the estimation of MDA and TAS.

Biochemical Estimations

Plasma MDA, as an index of lipid peroxidation, was estimated by the thiobarbituric-acid (TBA) reaction, in which MDA reacts with TBA under acidic, heated conditions to form a coloured adduct measured spectrophotometrically; results are expressed in nmol/ml [7]. Total antioxidant status was measured by a standardised assay of the reducing/radical-quenching capacity of plasma and expressed in mmol/L [12,13]. All assays were performed in duplicate with appropriate quality controls.

Statistical And Chronobiological Analysis

Data are presented as mean ± standard deviation (SD). Differences between patients and controls at each sampling time were

assessed by Student's *t*-test for independent samples (or the Mann–Whitney *U* test where appropriate); a two-tailed *p*-value below 0.05 was considered statistically significant. Circadian rhythmicity of MDA was evaluated by single-component (single-cosinor) rhythmometry, in which a twenty-four-hour cosine curve is fitted to the data by least squares, yielding the MESOR (rhythm-adjusted mean), the amplitude (half the peak-to-trough difference of the fitted curve) and the acrophase (clock time of the fitted maximum); the statistical significance of the fitted rhythm was determined for each group [22,23].

Ethical Considerations

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Ethical approval was obtained from the Institutional Ethics Committee of Index Medical College, Hospital & Research Centre / Malwanchal University prior to commencement, and written informed consent was obtained from every participant.

RESULTS

Participant Characteristics

Thirty patients with alcoholic hepatitis (mean age 42.6 ± 9.8 years; 28 men, 2 women) and thirty healthy controls (mean age 41.2 ± 8.7 years; 27 men, 3 women) completed the study. Participants ranged in age from 18 to 70 years. There was no statistically significant difference between the groups in age or sex distribution ($p > 0.05$), indicating that the groups were comparable with respect to these variables (Table 1).

Table 1 Demographic Characteristics of the Study Participants

Characteristic	Controls (N = 30)	Alcoholic Hepatitis (N = 30)	*P*-Value
Age (years, mean ± SD)	41.2 ± 8.7	42.6 ± 9.8	> 0.05
Sex (male / female)	27 / 3	28 / 2	> 0.05
Age range (years)	18–70	18–70	—

Circadian Profile of Plasma Malondialdehyde

Plasma MDA concentrations were significantly higher in patients than in controls at every sampling time (all $p < 0.001$; Table 2). In controls the concentration rose from a trough at 06:00 h to a peak at 18:00 h, whereas in patients the highest value occurred at 12:00 h.

Table 2 Plasma MDA Concentration (Nmol/Ml) At Four Circadian Sampling Times (Mean ± SD)

Time (H)	Controls (N = 30)	Alcoholic Hepatitis (N = 30)	*P*-Value
06:00	2.85 ± 0.42	4.92 ± 0.78	< 0.001
12:00	3.41 ± 0.51	5.87 ± 0.91	< 0.001
18:00	4.12 ± 0.58	5.21 ± 0.85	< 0.001
00:00	3.18 ± 0.47	4.65 ± 0.72	< 0.001

Single-cosinor analysis confirmed a statistically significant twenty-four-hour rhythm of MDA in both groups (rhythm-detection $p < 0.001$ in controls and $p < 0.01$ in patients; Table 3). In controls the acrophase occurred in the late afternoon (approximately 16:21 h). In patients the MESOR was significantly higher than in controls (5.16 ± 0.71 versus 3.39 ± 0.38 nmol/ml; $p < 0.001$), the amplitude was significantly lower (0.41 ± 0.12 versus 0.64 ± 0.15; $p = 0.002$), and the acrophase was advanced towards midday (12.18 ± 1.5 versus 16.35 ± 1.2 h; $p < 0.001$). The MDA rhythm in patients was thus characterised by a higher rhythm-adjusted mean, a smaller amplitude and an earlier peak (Figure 1).

Table 3 Cosinor Parameters of Plasma MDA

Parameter	Controls	Alcoholic Hepatitis	*P*-Value
MESOR (nmol/ml)	3.39 ± 0.38	5.16 ± 0.71	< 0.001
Amplitude (nmol/ml)	0.64 ± 0.15	0.41 ± 0.12	0.002
Acrophase (h)	16.35 ± 1.2	12.18 ± 1.5	< 0.001
Rhythm detection (*p*)	< 0.001	< 0.01	—

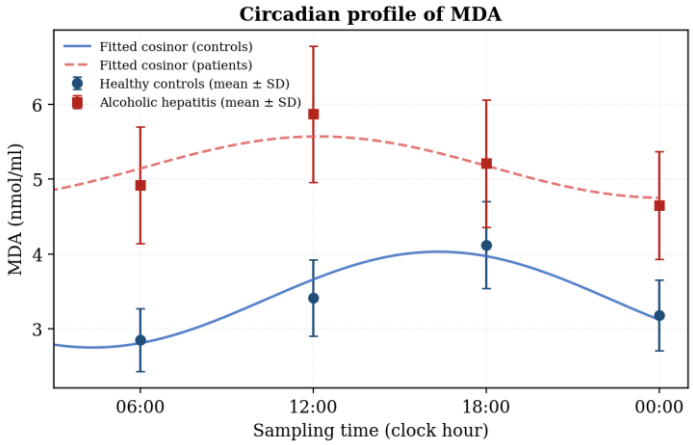

Figure 1. Circadian profile of plasma malondialdehyde (MDA) in controls and patients with alcoholic hepatitis across the four sampling times. Points represent group means and error bars the standard deviation; the fitted single-component cosine curves are superimposed.

Total Antioxidant Status

Plasma TAS was significantly lower in patients than in controls at every one of the four sampling times (all $p < 0.001$; Table 4). In controls TAS was highest at 06:00 h and lowest at 18:00 h; in patients the values were uniformly depressed and the diurnal variation was blunted. Expressed as the arithmetic mean of the four sampling times (a descriptive summary only, not a cosinor MESOR), TAS was 1.54 mmol/L in controls and 1.05 mmol/L in patients (Figure 2).

Table 4 Plasma Total Antioxidant Status (TAS, Mmol/L) At Four Circadian Sampling Times (Mean ± SD)

Time (h)	Controls (n = 30)	Alcoholic hepatitis (n = 30)	*p*-value
06:00	1.68 ± 0.21	1.12 ± 0.18	< 0.001
12:00	1.55 ± 0.19	1.05 ± 0.16	< 0.001
18:00	1.42 ± 0.17	0.98 ± 0.15	< 0.001
00:00	1.51 ± 0.18	1.03 ± 0.16	< 0.001

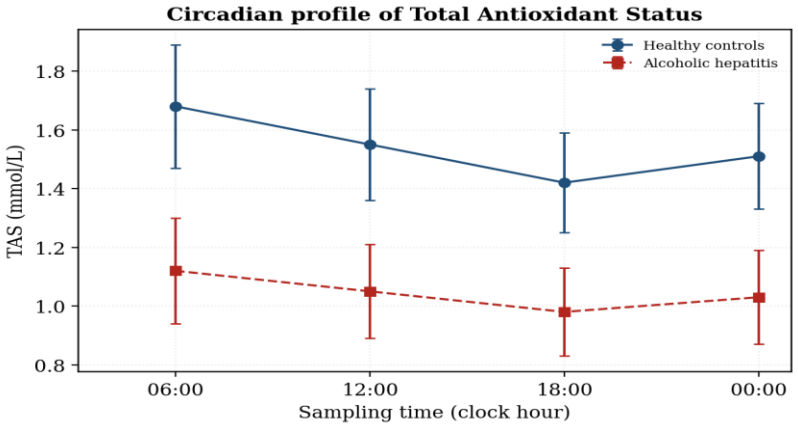

Figure 2. Circadian profile of plasma total antioxidant status (TAS) in controls and patients with alcoholic hepatitis. Points represent group means and error bars the standard deviation.

DISCUSSION

The principal findings of this study are that patients with alcoholic hepatitis exhibit, across the whole twenty-four-hour cycle, a substantially elevated rhythm-adjusted mean of plasma MDA together with a reduced amplitude and an advanced acrophase of its rhythm, and a persistently depressed total antioxidant status. Considered together, these observations indicate that lipid peroxidation in

alcoholic hepatitis is not only quantitatively increased but also temporally reorganised, and that the integrated antioxidant capacity is reduced at every time of day.

The elevation of MDA is consistent with the well-established enhancement of lipid peroxidation in ALD reported in Indian and international studies alike [8,9,10,11]. The novel contribution here is the temporal characterisation. The higher MESOR indicates that the average oxidative burden borne by patients is raised throughout the day and night rather than at any single time, while the reduced amplitude suggests a loss of the normal rhythmic modulation of lipid peroxidation. The advance of the acrophase from the late afternoon in controls towards midday in patients indicates a phase shift in the timing of peak lipid peroxidation. Such a change in phase would be entirely undetectable by the single-time-point designs that dominate the existing literature, and it may contribute to the apparent inconsistencies between earlier studies that sampled at different, and often unrecorded, clock hours [19,20]. Our findings are in keeping with earlier chronobiological work reporting altered rhythms of circulating lipid peroxides and antioxidants in cirrhosis and in other disease states [21,24].

The persistently reduced TAS complements the MDA findings by demonstrating that the integrated, largely non-enzymatic antioxidant buffering capacity of plasma is diminished at every sampling time in patients. Because TAS integrates the contributions of many antioxidants, its uniform depression indicates a globally weakened defence rather than an isolated deficiency, consistent with reports of reduced antioxidant capacity in ALD [11]. The blunting of its diurnal variation parallels the reduced amplitude of the MDA rhythm and suggests a broader flattening of the normal temporal organisation of redox balance in these patients.

Several mechanisms may plausibly underlie these observations, although the present cross-sectional design does not permit any of them to be established. Chronic ethanol exposure induces cytochrome P450 2E1 and impairs mitochondrial function, increasing ROS generation, while depleting glutathione and other antioxidants; the resulting sustained pro-oxidant state could raise the mean level of lipid peroxidation and lower antioxidant capacity [4,5,6]. Chronic alcohol consumption also disrupts circadian organisation — altering clock-gene expression in peripheral tissues, disturbing sleep and feeding, and suppressing melatonin secretion — which could plausibly account for the reduced amplitude and shifted phase of the MDA rhythm and for the blunting of the TAS variation [15,16,18]. The reciprocal coupling between the molecular clock and the redox state provides a conceptual framework within which increased oxidative stress and disturbed rhythmicity might reinforce one another [14,17]. These mechanisms are advanced as hypotheses consistent with the data, not as conclusions proven by them.

The findings have potential implications. For laboratory medicine, they reinforce the importance of standardising and recording the time of sampling when oxidative-stress markers are measured, and they raise the possibility that rhythm parameters, rather than single values, might provide more informative indices of redox disturbance [19,20]. More speculatively, a knowledge of the times of peak oxidative burden and of lowest antioxidant capacity could provide a rational, hypothesis-generating basis for the future investigation of chronotherapeutic strategies — the appropriately timed administration of antioxidant or hepatoprotective agents. It must be emphasised that the demonstration of a circadian pattern cannot by itself establish that timed intervention is beneficial; this remains a hypothesis for prospective interventional testing.

This study has limitations. It is cross-sectional and observational, and therefore cannot establish causation or the direction of any relationship. The sample size is modest, and the four sampling times, while sufficient to fit a single-cosinor model, limit the resolution with which the rhythm parameters can be estimated. Clock-gene expression and melatonin were not measured, so the proposed link to circadian disruption remains inferential. Sampling over a single twenty-four-hour cycle does not capture between-day variability. These limitations indicate the need for larger, longitudinal studies with denser sampling and concurrent measurement of chronobiological markers.

CONCLUSION

Patients with alcoholic hepatitis demonstrate an elevated, phase-advanced and amplitude-attenuated circadian rhythm of plasma lipid

peroxidation, together with a total antioxidant status that is significantly and persistently reduced across the whole twenty-four-hour cycle. These findings indicate that oxidative injury in alcoholic hepatitis is both quantitatively increased and temporally dysregulated. As a cross-sectional study, this work cannot establish causation or therapeutic efficacy; its value is in demonstrating the time-dependence of these markers and in generating hypotheses for the time-qualified interpretation of oxidative-stress measurements and for future chronotherapeutic research.

Declarations

Ethics Approval and Consent to Participate: The study was approved by the Institutional Ethics Committee of Index Medical College, Hospital & Research Centre / Malwanchal University. Written informed consent was obtained from all participants.

Consent For Publication: Not applicable

Availability of Data and Materials: The de-identified data supporting the findings are available from the corresponding author upon reasonable request, subject to institutional policy.

Competing Interests: The authors declare that they have no competing interests.

Authors' contributions: [e.g. SKS conceived and conducted the study, performed the assays and drafted the manuscript; SN supervised the work and critically revised the manuscript. Both authors approved the final version.]

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Related Work: This study forms part of a doctoral research programme on the circadian nature of oxidative-stress markers and antioxidant enzymes in alcoholic hepatitis; the circadian behaviour of the enzymatic antioxidant defences (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) in the same cohort is reported separately.

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