Decreased Melatonin Secretion is Associated with Increased Intestinal Permeability and Marker of Endotoxemia in Alcoholics

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ABSTRACT

Background: Chronic heavy alcohol use is known to cause gut leakiness and alcoholic liver disease (ALD), but only 30% of heavy drinkers develop increased intestinal permeability and ALD. The hypothesis of this study was that disruption of circadian rhythms is a potential risk factor in actively drinking alcoholics for gut leakiness and endotoxemia. Methods: We studied 20 subjects with alcohol use disorder (AD) and 17 healthy controls (HC, 6 day workers, 11 night workers). Subjects wore a wrist actiwatch for 7 days and underwent a 24 hour dim light phase assessment and urine collection for intestinal permeability. Results: The AD group had significantly less total sleep time, and increased fragmentation of sleep (p<0.05). AD also had significantly lower plasma melatonin levels compared to the HC (mean AUC 322.78 ± 228.21 versus 568.75 ± 304.26 pg/ml, p=0.03). In the AD group, AUC of melatonin was inversely correlated with small bowel and colonic intestinal permeability (L/M ratio, r= -0.39, p=0.03; urinary sucralose, r= -0.47, p=0.01). Cosinor analysis of lipopolysaccharide binding protein (LBP; marker of endotoxemia) and lipopolysaccharide (LPS) every 4 h for 24 h in HC and AD subjects had a mesor of 5026.15 ± 409.56 vs 6818.02 ± 628.78 ng/ml (p<0.01) and 0.09 ± 0.03 vs 0.15 ± 0.19 EU/ml (p< 0.05), respectively. Conclusions: We found plasma melatonin was significantly lower in the AD group and lower melatonin levels correlated with increased intestinal permeability and a marker of endotoxemia. Our study suggests the suppression of melatonin in AD may promote gut leakiness and endotoxemia.
INTRODUCTION

Alcohol is consumed by about half of the US population, and is the most frequently abused drug in the world (31). However, the most serious complication of heavy drinking, alcoholic liver disease (ALD), remains only partially understood. The amount and duration of alcohol consumed appear to be the most important risk factors for the development of ALD. About 20 to 30% of individuals who consume over 30 grams/day for 10 years go onto develop cirrhosis (46). However, there does not appear to be a clearly linear dose effect above this threshold as certain groups of extremely heavy drinkers (> 120g/day) developed cirrhosis at a low rate (18.5%) (5). Therefore, epidemiologic studies have shown that alcohol is a necessary cofactor to cause clinically significant ALD and cirrhosis, but other additional co-factors are required.

The most well supported pathogenesis of ALD is that alcohol increases intestinal permeability which increases gut derived endotoxin, such as lipopolysaccharide (LPS), in the blood. This increase in gut derived endotoxin initiates endotoxin-mediated hepatocellular damage and other alcohol-induced, inflammation mediated, tissue injury and organ failure. LPS causes damage by activating the innate immune system via TLR4, which stimulates NADPH oxidase and causes an increase in reactive oxygen species (ROS) and inflammatory cytokines through NFκB and EGR-1 dependent pathways (49). The role of this pathway has been supported by a number of experimental findings including: 1) eliminating bacteria in the gut prevents endotoxemia and ALD in rats (1); 2) alcohol causes hyperpermeability in rats which precedes the development endotoxemia and ALD (19); and 3) gut leakiness is present in 80% of human alcoholics with ALD but not in alcoholics without liver injury (21, 33, 35).

Yet, not all alcoholics develop gut leakiness and endotoxemia. Identifying other risk factors for increased intestinal permeability related to heavy alcohol consumption is important to identify individuals at risk for clinically significant ALD and to better understand alcohol related mechanisms of liver injury. The finding that clock genes are present in the epithelial cells of the intestinal tract (18, 41) makes it highly plausible that the disruption of circadian homeostasis could be a cofactor and negatively impact intestinal function, such as intestinal barrier. Indeed several inflammatory GI and systemic disorders are more common in shift workers who have disrupted circadian rhythms such as peptic ulcer disease (34), colon cancer (22), and irritable bowel syndrome (29). Taken together, these epidemiological and experimental findings suggest that disruption of circadian rhythms, which synchronize an organism’s internal physiology to the external environment, could impact intestinal barrier function.

Whether circadian desynchrony is a cofactor in alcohol induced intestinal hyperpermeability is a gap in our current knowledge.

In humans, the central circadian clock is located in the suprachiasmatic nucleus (SCN), which during the biological night triggers the pineal gland to secrete melatonin. Thus the measurement of melatonin rhythm is a commonly used method to assess central circadian timing in humans (6). A method of assessing integrity of peripheral circadian homeostasis is the measurement of core circadian genes such as CLOCK and PER genes in circulating blood monocytes or tissue clock genes in intestine or liver. Our recent work showed that alcohol increased circadian clock genes, CLOCK and PER2, in intestinal epithelial cells, CaCo-2 cells, and blocking CLOCK or PER2 with
SiRNA prevented alcohol induced hyperpermeability (44). Our subsequent studies in mice have shown that circadian disruption, either environmental or genetic, increases alcohol induced intestinal permeability and liver injury (43). Therefore in both in vitro and in vivo models circadian homeostasis appears critical to intestinal barrier function in response to an injurious agent like alcohol. However, the relationship between alcohol induced hyperpermeability and central circadian rhythms in humans has not been studied.

We hypothesized that the disruption of circadian rhythms in alcoholics is a co-factor that promotes gut leakiness to endotoxin. Accordingly, the goals of the current study were to: 1) to determine if alcoholics have altered central circadian rhythms, as measured by plasma melatonin phase and amplitude over 24 hours compared to nonalcoholic healthy controls; and 2) to determine if alcoholics with circadian misalignment and/or altered melatonin secretion have increased intestinal permeability and endotoxemia compared to AD without increased intestinal permeability, as assessed by correlating markers of intestinal permeability / endotoxemia and marker of circadian homeostasis. We measured intestinal permeability by 24 hour urinary sugar probes (sucrose, lactulose/mannitol, and sucralose) and assessed endotoxin exposure by plasma lipopolysaccharide (LPS) and serum Lipopolysaccharide-Binding Protein (LBP), which is an acute phase protein produced by the liver and binds to the endotoxin LPS.

**Methods**

**Participants**

Participants were recruited through a combination of advertisements, posted flyers, the Rush University Medical Center GI clinic, or self-referral. Alcohol Use Disorder (AD) was defined by the Lifetime Drinking History (LDH) questionnaire as previously reported (40). Controls (HC) were healthy shift workers (primarily nurses) who did not fulfill the criteria for AD per LDH questionnaire, and had never been a daily drinker. The work schedule for controls was either day or night shift. We elected to study both day and night workers because it is well-established that a subset of AD have abnormal sleep patterns (8), and thus the healthy night workers represented a control group with disrupted sleep. All subjects had been on a stable work schedule for three months. All subjects completed blood tests and questionnaires at their initial visit and were excluded if they met any of the following criteria: 1) Unreliable drinking history; 2) Clinically detectable liver disease defined as abnormal LFTs or any history of ascites, jaundice, or variceal bleeding; 3) Renal impairment (creatinine>1.2 mg/dL), 4) AD positive for other markers of liver disease such as smooth muscle antibody, hepatitis B surface antigen or hemochromatosis; 5) Major depression (score ≥ 15 or any endorsement of suicidal intent on the Beck Depression Inventory)(4); 6) Sleep apnea (score high risk ≥ 2 or more categories on the Berlin Questionnaire)(28), 7) Restless leg syndrome IRLSSG consensus criteria for Restless Leg Syndrome, 8) Insulin-requiring diabetes and/or uncontrolled diabetes (Hgb-A1c>8%); 9) Significant peripheral edema; 10) Sepsis; 11) Clinically significant cardiac failure (NY classification stage III/IV); 12) Patients with low platelet counts (<80k), uncorrectable prolonged prothrombin time (>15 sec); 13) Infection in the previous month, recent history of antibiotic use (within past 4 weeks);
14) Regular use of medications that affect intestinal permeability, intestinal motility and/or endogenous melatonin including metoclopramide, NSAIDs, beta blocker, psychotropic medication, hypnotics and exogenous melatonin products during 4 weeks prior to the study; 15) Asians due to the possible confounding effect of a different polymorphism of enzymes involved in alcohol metabolism.

**Baseline Measures**

**The Pittsburgh Sleep Quality Index**

The Pittsburgh Sleep Quality Index (PSQI) is a 19 item self-administered questionnaire of 7 components (10). Overall scores ranged from 0 to 21 with lower scores indicating better sleep.

**Lifetime Drinking History**

The Lifetime Drinking History (LDH) is a structured interview that is designed to provide quantitative indices of an individual's alcohol consumption patterns from the onset of regular drinking. Attention is focused on quantity, frequency, variability in consumption, types of beverages, and life events that mark a change in drinking pattern. Solitary versus social drinking, and time of day when alcohol is consumed are also recorded. The interview takes approximately 20 to 30 minutes to complete and has been shown to be a validated measure of alcohol consumption (23).

**Sleep Schedule**

After a baseline questionnaire assessment and blood tests, all subjects were asked to keep their usual sleep schedule for the week prior to the 24 hour circadian phase assessment in the Biological Rhythms Research Laboratory. All subjects kept a sleep diary, a food diary, and wore a wrist monitor (30 second epochs, Spectrum Actiwatch, Phillips, Inc) on their nondominant wrist for 7 days prior to the phase assessment. Actigraphy data was scored for sleep intervals and variables of interest were calculated by Actiware 5.70.1 program (Respirationics, Bend, OR). Actigraphic derived sleep indices included sleep duration (or total time in bed), wake after sleep onset, total sleep time, and sleep percentage.

**Melatonin Assessment and Analysis**

Plasma melatonin was collected hourly for 24 hours between 13:00 and 12:00 h, for a total of 24 samples. Patient were kept in dim light (<5 lux, measured every 2 hours with a Minolta TIL-1 light meter) and seated in a recliner chair in the Biological Rhythms Research Laboratory. After 30 minutes in dim light, a 3ml blood sample was collected every 60 minutes. An intravenous (IV) line was placed to avoid multiple needle sticks, and the IV was kept patent by a slow drip (10 cc/hr) of heparinized saline, 7500IU/L in half normal saline. In order to prevent sample dilution, before collecting a blood sample through the plastic tubing, 2 ml of the saline solution admixed with blood was removed via a 3 way stopcock system and returned to the patient after the sample was drawn. During the session, subjects were kept awake, watched a dim TV, or talked to each other. After collection, the samples were centrifuged at 4 C and 1000 rpm for 15 minutes. The plasma was then frozen and shipped on dry ice to SolidPhase, Inc.
(Portland, ME) using the Bühlmann Direct Plasma Melatonin RIA kit (ALPCO Diagnostics, Windham, NH), which has an analytical sensitivity of 0.2 pg/mL. Inter-Assay variability ranged from 7.9 to 11.7%.

The peak value or maximum point of each melatonin profile was identified. Additionally, area under the curve (AUC) was calculated for each melatonin profile using the trapezoidal method (38). The dim light melatonin onset (DLMO) was calculated as the point in time when the melatonin concentration exceeded and remained above the threshold for 1 hour. The threshold was calculated from the mean of 3 low consecutive daytime values plus twice the standard deviation of these points as previously reported (48).

### Intestinal Permeability Measurement

Ingestion of sugar probes, or large and difficult to absorb sugars, is a common method to measure intestinal permeability in vivo that has been have previously used in humans (16). After a 4 hour fast, each subject ingested 300ml of liquid containing 7.5 grams of lactulose, 2 grams of mannitol, 40 grams of sucrose, and 2 grams of sucralose. Before ingestion of the sugar probes, each subject was asked to empty his bladder completely. Thereafter, all urine was collected for 24 hours in first 5 hours, next 7 hours, and last 12 hour aliquots. Subjects were not allowed to eat for 4 hours after the start of the urine collection. Urine volumes were recorded and aliquots of urine were stored at -80°C until analysis. Measurement of urinary sugars was done by gas chromatography and calculated as percent excretion of oral intake. 5 hour urinary sucrose excretion is primarily a marker of gastroduodneal permeability, 5 hour urinary lactulose, mannitol and lactulose/mannitol ratio (L/M) are primarily markers of small bowel permeability, and 24 hour urinary sucralose and lactulose excretion are markers of total gut permeability with sucralose primarily representing colonic permeability (2). This is due to both sucralose and lactulose being able to permeate through both the small and large intestine (colon). However, sucralose is not fermented by colonic bacteria while ~75% of lactulose and mannitol are fermented by colonic bacteria (25).

### Lipopolysaccharide-Binding Protein

Lipopolysaccharide binding protein (LBP)(ng/ml) was determined from blood samples collected every four hours and was measured in serum using an ELISA kit from Cell Sciences Inc (catalogue # HK315).

### Lipopolysaccharide Measurement

Lipopolysaccharide (LPS) (EU/ml) was determined from blood samples collected every four hours and was measured in plasma citrate using a Kinetic Turbidmetric LAL assay the PYROGENT™-5000 from Lonza (catalogue #N383).

### Statistical Analysis

Chi-square tests, independent t-test, and Mann-Whitney U test were used to compare the two subject groups. To assess the relationship between melatonin and intestinal permeability, different parameters of melatonin (AUC, DLMO) were analyzed by linear regression. The LBP and LPS levels were taken at 6 times points and were analyzed by cosine rhythmometric analysis (cosinor) as previously described. (27)(13) To
access differences in rhythmicity the mesor (Midline Estimating Statistic of Rhythm), amplitude, and acrophase were calculated for each 24 hour period. All statistical analysis were two tailed, statistical significance was determined by using a \( p \)-value of <0.05. Statistics were performed using SPSS version 19.0 or R (v. 3.8.1) in the cosinor package.

Results

Subjects

A total of 56 subjects were consented for the trial, and 37 participated. The enrollment and outcomes for subjects that did not participate are shown in Figure 1. The main reason subjects did not participate after being screened was they were lost to follow up or voluntarily withdrew.

Clinical Variables

The baseline characteristics were different between the two groups and are outlined in Table 1. AD subjects were more likely to be older, male, African American, have a higher BMI, have less education, and were could be unemployed. The AD group also reported a poorer sleep quality by PSQI as compared with HC, 7.2 ± 3.8 vs 4.6 ± 2.9 (p=0.03). AD subjects had a higher BDI score for depression (9.5 ± 6.5 vs 3.5 ± 4.1, \( p<0.01 \)) but it is important to note that patients with major depression defined as BDI score of >15 were excluded.

Actigraphy Data

Actigraphy derived sleep parameter data is shown in Table 2. In AD, 1 subject was excluded for actiwatch failure and three other subjects were not compliant with wearing the actiwatch for the full week. In the remaining 16 subjects with AD, sleep duration (time in bed) and total sleep time were significantly lower than HC – 404.8 ± 67.3 vs 462.5 ± 45.5.2 and 340.3 ± 60.7 vs 409.6 ± 45.1; respectively (\( p < 0.01 \)). In addition, AD subjects had more fragmented sleep – 27.7 ± 9.1 vs 16.9 ± 4.10 compared to HC (\( p < 0.01 \)). In addition, there was no difference in the control group day workers and night workers which is also shown in Table 2.

Intestinal Permeability data between Groups

There was no difference in intestinal permeability from day workers or night workers by 5 hour sucrose, 5 hour lactulose/mannitol ratio, or 24 hour sucralose with \( p \)-values of 0.21, 0.37, and 0.18, respectively. Figure 2 shows the difference in gastroduodenal (2A), small bowel (2B), and whole gut/colonic permeability (2C) between the AD and HC group with 0.45 ± 0.29 vs 0.46 ± 0.23 (\( p=0.53 \)), 0.05 ± 0.06 vs 0.13 ± 0.18 (\( p=0.01 \)), and 0.84 ± 0.48 vs 1.41 ± 1.43 (\( p=0.08 \)) for 5 hour sucrose, 5 hour lactulose/mannitol ratio, and 24 hour sucralose respectively with all values as % excretion of oral dose. The range for the 5 hour lactulose, 5 hour lactulose/mannitol ratio, and 24 hour sucralose was 0.78, 0.65, and 6.08, respectively. There was not a statistical difference between either 24 hour urinary lactulose or the second 12 hour urinary sucralose excretion at 5.40 ± 6.55 vs 6.24 ± 5.57 (\( p=0.11 \)) and 0.12 ± 0.22 vs 0.20 ± 0.18 (\( p=0.09 \)), respectively. It is important to note that our AD group excluded subjects with liver disease, so not all the
Comparison of Melatonin Profiles between Groups
The mean DLMO for the AD group was significantly earlier than the HC group (19:58 ± 1.18 h versus 21:03 ± 3.00 h, p=0.04). This difference was found however mainly between the AD group and the night workers with a later DLMO in the night workers group. The night workers had a mean DLMO of 21:29 ± 3:25 h and the day workers were 20:38 ± 1:52 h. The DLMO was not statistically different (p=0.30) between the AD group and the HC day workers while it was when compared to the night shift workers (p=0.03). The AUC of the plasma melatonin profiles in the AD and HC groups are shown in Figure 3. There was significantly lower plasma melatonin AUC in AD subjects compared to HC, 322.78 ± 228.21 pg/ml/hr vs 568.75 ± 304.26 pg/ml/hr (p=0.03). The mean AUC for Day subjects was 401.38 ± 100.27 pg/ml/hr and for night workers was 661.13 ± 342.17 pg/ml/hr, which was not statistically different (p=0.13). One AD subject’s data was excluded as they lost their IV and it could not be successfully replaced. Similarly there was a significant decrease in median peak melatonin amplitude in AD subjects compared to HC – 41.41 pg/ml ± 30.09 vs 70.81 ± 41.41 (p=0.04). The AUC of melatonin in the control group did not significantly correlate with age (r= 0.10, p=0.20), but the AUC of melatonin in AD group was significantly correlated with age – (r=0.64, p=0.01).

Correlation between Intestinal Permeability and Melatonin
Figure 4 shows a linear regression analysis of AUC of plasma melatonin compared to 5 hour urinary L/M ratio (4A) and 24 hour urinary sucralose (4B). There was a statistically significant negative correlation of both small bowel and whole gut/colonic permeability at r= −0.39, p=0.03 and r= −0.47, p=0.01, respectively. This analysis excluded 8 subjects – 3 in the AD group and 1 in the control groups who were extremely leaky with > 2 x SD of mean sugar probe excretion, 3 in the control group with melatonin AUC > 2 x SD of mean, and one subject who lost their IV. Of the subjects with markedly increased permeability, two had a BMI of 40, and one was a poly-substance abuser.

Correlation between Actigraphy and Intestinal Permeability or Melatonin
There was no correlation by linear regression between whole gut permeability or 24 hour urinary sucralose and actigraphy measures (sleep duration, sleep fragmentation, and sleep percentage) with a p=0.76, p=0.27, and p=0.56, respectively. Similarly there was no correlation found between small bowel permeability or L/M ratio and actigraphy markers (sleep duration, sleep fragmentation, or sleep percentage) with a p-value of 0.62, 0.21, and 0.78 respectively. Melatonin AUC also did not show a correlation between sleep duration – p=0.44, fragmentation – p=0.60, or sleep percentage – p=0.37.

Correlation between Intestinal Permeability and Markers of endotoxemia
(Lipopolysaccharide Binding Protein and Lipopolysaccharide)
To determine whether there is any relationship between changes in intestinal permeability and endotoxemia, we performed a correlation analysis. By linear
regression, the mean LBP levels did correlate with urinary sucralose in the AD group, 
\( r=0.50, \ p=0.01 \) as seen in Figure 4C, but LBP did not correlate with urinary L/M ratio 
\( p=0.63 \). This is not surprising as bacteria levels are higher in the colon than small 
bowel, so the colon should be a greater source of endotoxemia. Mean LPS levels did 
not correlate with intestinal permeability by 24 hour sucralose \( p=0.20 \) or L/M ratio 
\( p=0.58 \). The LBP and LPS levels at 6 different time points were measured in 19 of 20 
AD subjects and 14 of 17 HC subjects. There was no significant difference between 
mean LBP levels or LPS levels in the AD and HC group, at 5026.15 ± 2979.01 and 
6818.02 ± 4402.26 ng/ml \( p=0.20 \) for LBP and 0.10 ± 0.10 and 0.11 ± .15 EU/ml for 
LPS. However, linear statistical analysis is not appropriate to analyze readouts with 
circadian oscillation and a cosinor analysis can test the rhythm of biological measures 
over a period of time (in this case 24 hours). The mesor, amplitude and acrophase 
(duration of one cycle) were calculated from a cosinor regression model. The results of 
the cosinor model are shown in Figure 5. For LBP, in the AD and HC group the mesor, 
amplitude, and acrophase of the model was 6818.02 ± 628.78 vs 5026.15 ± 409.56 
\( p<0.01 \), 549.12 ± 579.02 vs 485.23 ± 674.75 \( p=0.47 \), and 0.35 ± 1.06 vs 1.05 ± 1.39 
\( p=0.44 \), respectively. For LPS, in the AD and HC group the mesor, amplitude, and 
amplitude of the model was 0.15 ± 0.19 vs 0.09 ± 0.03 \( p<0.05 \), 0.01 ± 0.03 vs 0.02 ± 
0.03 \( p=0.68 \), and -0.75 ± 1.88 \( p=0.75 \), respectively. The mesor, or Midline 
Estimating Statistic of Rhythm, which is an estimate of central distribution or mean of an 
ocillating variable was the only value that was significantly different between the two 
groups.

**Discussion**

The hypothesis of the current study was that AD would have altered central 
circadian rhythms as measured by 24 hour plasma melatonin profile and that circadian 
disruption is a key cofactor in alcohol induced intestinal permeability. Markers of 
intestinal permeability, L/M ratio (small bowel) and sucralose (whole gut/colonic), are 
known to be increased in AD (20), but if our hypothesis is correct, subjects with 
abnormal melatonin profiles would be more likely to develop gut leakiness. Indeed, we 
found the AUC of plasma melatonin was significantly lower in the AD group, and that 
there was an inverse correlation between AUC and both small bowel and colonic 
testintestinal permeability. In addition, a marker of endotoxemia, LBP, correlated with 
intestinal permeability, possibly reflecting the impact of systemic inflammation from gut 
leakiness. This data suggests melatonin may be an effective marker to help determine 
which AD subjects have disrupted circadian homeostasis and are at risk for intestinal 
hyperpermeability, endotoxemia, liver disease, and perhaps other alcohol associated, 
inflammatory mediated, tissue injury. To our knowledge, this is the first study to report 
this finding.

For our control group, we chose day workers and also night workers as a control 
group with sleep disruption as actively drinking AD are known to have disrupted sleep 
(7, 8, 17). Despite including night shift workers, the AD group still had significantly more 
disrupted sleep than the control group by wrist actigraphy as assessed by sleep 
fragmentation, sleep duration, and sleep percentage. Our control group therefore still 
had less disordered sleep that our AD group. We did find a difference in DLMO
between the AD and HC participants, however, this finding was present only in comparison to night shift workers in the HC group which would be expected. There was no statistically significant difference between the DLMO of day workers and the AD group. The finding that AD group did not have a significant difference in DLMO compared to controls is consistent with another previous large human study (12). Our finding that AD had lower plasma melatonin secretion measured by AUC or peak melatonin is novel.

Low melatonin may indicate disrupted circadian homeostasis, and low secretion of melatonin has also been associated with diabetes (24), obesity (45), Parkinson’s disease (47), and aging (32). Melatonin is a powerful antioxidant, and is known in multiple animal models to be protective of colitis and intestinal hyperpermeability (26, 30) including ethanol induced intestinal hyperpermeability (42). Our finding is also supported by our animal data that alcohol fed rodents that have circadian alteration induced either environmentally (light/dark phase shift) or by genetic manipulation (clock mutant) have increase alcohol induced hyperpermeability and liver pathology (43).

There are a number of important limitations to the present study. Our AD group had more men, more African American subjects, and was older compared to the HC group. The impact of age and gender on melatonin levels is controversial. Salivary melatonin was not found to decrease with age or gender in one large healthy control cohort (9), but other groups have reported that melatonin levels decreased with age in middle aged subjects (51). In another study comparing young subjects to subjects over age 65, salivary melatonin was found to be decreased by 37% ± 12.5% in men but was not significantly lowered in women (50). The age difference in our study was under 20 years and we included no subjects over the age of 65. In addition, shift work could also affect melatonin, but the years of shift work were not associated with melatonin levels in the NHS study (39). It is therefore unlikely that our findings of differences in plasma melatonin AUC can be explained by differences in age, gender, or shift work between AD and HC groups but this study is unable to definitely answer that question which is a limitation. Finally, it is important to note that cirrhotic AD were excluded from the present study. This was due to changes in melatonin amplitude or phase that can occur with end organ damage like cirrhosis which has been demonstrated previously in hepatic encephalopathy (11), but limit the ability of this study to make any definitive conclusions about subjects with ALD.

There is other data to support alcohol decreasing melatonin. In two studies acute alcohol intake at night was associated with reducing melatonin levels in salivary melatonin (15) or plasma melatonin (37), but was not associated with a change in the timing or phase of melatonin as measured by DLMO. An additional study that evaluated two doses of acute alcohol consumption found a dose effect for alcohol to inhibit melatonin. (36) (35) In studies with AD subjects there were higher levels of melatonin during active drinking compared to levels during abstinence (17). The largest age and gender matched study comparing subjects with AD and healthy controls, found DLMO mildly delayed (0:40 h) by one but not by the other method of DLMO calculation (12). The key difference between the current study and the study by Conroy et. al. was our study was assessing active drinkers while Conroy studied subjects that were sober or in a recovery program for 3-13 weeks. Although our AD subjects were actively drinking, they were all breathalyzed on arrival and were negative for alcohol, so this appears to
be a carryover effect of recent alcohol consumption. Another key difference between
the present study and previous data on melatonin and alcohol was we used plasma
melatonin and not salivary melatonin, as levels of melatonin are 10 fold higher in the
plasma compared to saliva (14).

In our study we examined the endotoxin LPS, and a marker of endotoxemia,
LBP. LBP has been previously reported to be elevated in AD particularly during (19)
elevated in cirrhotics with increased permeability and bacterial translocation (37).
In our study, we did not find significantly elevated LBP levels or LPS levels in our AD
group compared to controls. However, when examining the timing of six different
measurements of LBP and LPS levels by a cosinor analysis (Figure 5), we found a
significantly different mesor between the two groups correlating with higher levels of
LBP and LPS in the AD group during the day. This is interesting as due to the robust
circadian rhythm of the immune system (41), relatively elevated levels of LPS would
normally be anticipated to be at night. The AD group had their highest levels of LBP and
LPS during the day which may further indicate a disruption of circadian homeostasis in
the peripheral organs like the intestine. The alteration in LBP and LPS we observed in
the AD group could be from a change in the rate of endotoxin leak from the intestine,
alteration in liver production of LBP, or decreased clearance. Further mechanistic
studies are needed to examine these possibilities and to correlate whether our finding
may be from loss of circadian homeostasis, alterations in melatonin, or sleep disruption.

In summary, the main finding of this study was low melatonin secretion correlates
with alcohol induced intestinal hyperpermeability. This novel finding could potentially
help to identify alcoholics at risk for intestinal leakiness, endotoxemia, ALD, or other
inflammation mediated, alcohol-induced tissue injury and organ failure. If confirmed, it
could offer risk stratification for clinicians accessing individuals with heavy drinking and
proposes possible chronotherapeutics for future investigations such as melatonin
supplementation in alcoholics at risk for ALD. Melatonin could be a marker of disrupted
central circadian homeostasis or could be acting locally as an antioxidant in the GI tract,
where levels are known to be 50 fold higher than in plasma. It is not well understood
how plasma levels of melatonin correlate with levels in the GI tract, as studies
comparing plasma and gastrointestinal levels of melatonin have not been done. Further
studies are needed to assess this question, and whether decreased plasma melatonin
AUC correlates with alterations in a peripheral circadian rhythms which could lead to
altered metabolism, tight junction protein function, increased intestinal permeability, and
inflammation.

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**FIGURE LEGENDS**

**Figure 1. Consort flow diagram of this study.** 56 research subjects were invited to participate in this study. Seven withdrew, six were lost to follow up, one was excluded because they failed to meet inclusion criteria (prohibited medication), and three had medical conditions that failed to meet the inclusion criteria. 37 study subjects completed all study assessments.

**Figure 2. Intestinal permeability in the Control and AD goups.** AD subjects and healthy controls were assessed for intestinal permeability using an oral sugar solution containing sucrose, lactulose, mannitol, and sucralse and GC analysis of 5 and 24 hour urine samples as described in Materials and Methods. A) 5 hour urinary sucrose and C) 24 hour urinary sucralse (markers of gastroduodenal and colonic permeability, respectively) were not significantly different in AD subjects compared to controls. B) 5 hour urinary lactulose and mannitol (L/M) ratio (markers of small intestinal permeability) was significantly higher in AD patients compared to controls. Data are presented as mean % urinary excretion of the oral dose in ± SE. Statistical significance = p<0.05.

**Figure 3. Comparison of Area Under the Curve of Melatonin in Controls and Alcoholics.** Blood was drawn every hour under dim light (< 5 lux) and plasma melatonin was measured by RIA as described in Materials and Methods. Melatonin Area Under the Curve (AUC) was calculated by trapezoidal method. Plasma Melatonin AUC was significantly low in AD subjects vs. healthy controls. Data are presented as a boxplot of AUC pg/ml/h.

**Figure 4. Intestinal Permeability Correlates with Plasma Melatonin AUC & LBP.** Plasma melatonin AUC measured in dim light by RIA and intestinal permeability as measured using an oral sugar solution containing sucrose, lactulose, mannitol, and sucralse after 24 hours by GC analysis were performed as described in the Materials and Methods section. Comparison by linear regression showed a significant negative correlation between Melatonin AUC in A) 5 h urinary L/M ratio (small bowel) and B) 24 h urinary sucralse (colonic) intestinal permeability. C) Serum levels of LPS binding protein (LBP), an indirect measure of systemic endotoxin exposure, positively correlated with urinary sucralse (colonic) permeability. All analysis was done by linear regression. A log transformation of L/M ratio was done due a positive skew of the data. Analysis excluded subjects with values > 2 SD which included 8 subjects – 7 with
elevated intestinal permeability and 1 with elevated melatonin AUC. 1 subject was excluded due to the loss of their IV.

**Figure 5. Vector Angle of LBP in Controls Compared to Alcoholics.**

Serum levels of LPS binding protein (LBP), an indirect measure of systemic endotoxin exposure (A) and plasma levels of LPS (B), were determined for AD subjects and healthy controls every 4 h over 24 h in dim light (<5 lux) at 1600, 2000, 2400, 0400, 0800, and 1200 hours, respectively by ELISA or Turbidmetric LAL assay as described in the Materials and Methods. For each subject, the 6 values were analyzed with a cosinor model that fits a single cosine wave function to the data with a period of 24 hours. From this model the mesor, amplitude, and acrophase were calculated. The fitted values are plotted in this figure. The p-value for the mesor, or Midline Estimating Statistic of Rhythm, which represents the mean distribution of the model is shown between the two groups – AD and HC.
Figure 1: CONSORT Flow Diagram

56 subjects recruited

30 met criteria for AD
- Excluded:
  - 4 Withdrew
  - 4 Lost to follow up
  - 1 Diabetes
  - 1 Chronic Pancreatitis

26 were HC without AD
- Excluded:
  - 1 Took antidepressant
  - 3 Withdrew
  - 2 Lost to follow up
  - 2 Could not get a reliable IV
  - 1 OSA

20 Participants with AD
- 11 worked Night Shift
- 6 worked Day Shift

17 Participants without AD
Figure 2: Intestinal Permeability in the Control and AD groups

A

5 hour Urinary Sucrose
(% Excretion of oral dose)

B

5 hour L/M ratio
(% Excretion of oral dose)

C

24 hour Urinary Sucrose
(% Excretion of oral dose)

p=0.60

p=0.01

p=0.08

Controls
Alcoholics
Figure 3: Comparison of Area Under the Curve of Melatonin in Controls and Alcoholics

AUC of Plasma Melatonin (pg/ml/h)

Controls

Alcoholics

p=0.03
Figure 4: Intestinal Permeability Correlates with Plasma Melatonin AUC & LBP

A

\[ r = -0.39; R^2 = 0.15; p = 0.03 \]

B

\[ r = -0.47; R^2 = 0.22; p = 0.01 \]

C

\[ r = 0.62; R^2 = 0.37; p = 0.01 \]
Figure 5 – Lipopolysaccharide (LPS) and LPS bind protein (LPB) in Alcoholic and Control subjects

A. Cosinor Model Plot of LBP levels in Alcoholics and Controls

B. Cosinor Model Plot of LPS levels in Alcoholics and Controls

- Alcoholic
- Control

P<0.01

P<0.05
## Table 1: Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>AD (N= 20)</th>
<th>HC (N= 17)</th>
<th>p-value (less than)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, Mean</strong></td>
<td>45.9</td>
<td>27.7</td>
<td><strong>0.00</strong></td>
</tr>
<tr>
<td><strong>Gender (male n; female n)</strong></td>
<td>15 M; 5 F</td>
<td>15 F; 2 M</td>
<td><strong>0.00</strong></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td>4 C; 16 AA</td>
<td>15 C; 2 L</td>
<td><strong>0.00</strong></td>
</tr>
<tr>
<td><strong>Education, median yrs</strong></td>
<td>14.0</td>
<td>16.0</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td><strong>Employment</strong></td>
<td>10 U; 10 E</td>
<td>15 E</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td><strong>Years of Heavy Drinking</strong></td>
<td>27.5</td>
<td>0</td>
<td><strong>0.00</strong></td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>28.9</td>
<td>24.5</td>
<td><strong>0.02</strong></td>
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<tr>
<td><strong>Average Drinks/week</strong></td>
<td>55.0</td>
<td>0</td>
<td><strong>0.00</strong></td>
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<tr>
<td><strong>Maximum Drinks/day</strong></td>
<td>10.5</td>
<td>1</td>
<td><strong>0.00</strong></td>
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<tr>
<td><strong>Days Drink/Month</strong></td>
<td>18.1</td>
<td>2</td>
<td><strong>0.00</strong></td>
</tr>
<tr>
<td><strong>Adverse Life Events Related to Alcohol; n, %</strong></td>
<td>13, 70%</td>
<td>0</td>
<td><strong>0.00</strong></td>
</tr>
<tr>
<td><strong>Tobacco Use, n, %</strong></td>
<td>9, 45%</td>
<td>2, 13%</td>
<td><strong>0.00</strong></td>
</tr>
<tr>
<td><strong>Illicit Drug Use, n, %</strong></td>
<td>8, 40%</td>
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<td><strong>0.00</strong></td>
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<tr>
<td><strong>PSQI</strong></td>
<td>7.2</td>
<td>4.6</td>
<td><strong>0.03</strong></td>
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<tr>
<td><strong>PIDS</strong></td>
<td>7.8</td>
<td>5.4</td>
<td><strong>0.10</strong></td>
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<td><strong>BDI</strong></td>
<td>9.5</td>
<td>3.5</td>
<td><strong>0.00</strong></td>
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<tr>
<td><strong>DLMO (hh:mm)</strong></td>
<td>19:58</td>
<td>21:03</td>
<td><strong>0.04</strong></td>
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<tr>
<td>Table 2: Actigraphy Data 7 Days Prior to Circadian Assessment</td>
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<tr>
<td>-------------------------------------------------------------</td>
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<tr>
<td>AD (N= 16)</td>
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</tr>
<tr>
<td>Mean</td>
<td>SD</td>
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<td></td>
</tr>
<tr>
<td>Sleep Duration (Time in Bed)</td>
<td>404.8</td>
<td>67.3</td>
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</tr>
<tr>
<td>HC (N= 17)</td>
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<td></td>
</tr>
<tr>
<td>Mean</td>
<td>SD</td>
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<td></td>
</tr>
<tr>
<td>p-value</td>
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<tr>
<td>p-value</td>
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<tr>
<td>Sleep Percentage</td>
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<tr>
<td>Fragmentation</td>
<td>27.7</td>
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<tr>
<td>Sleep Percentage</td>
<td>89.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragmentation</td>
<td>16.9</td>
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<tr>
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<td>&lt;0.00</td>
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HC Group

<table>
<thead>
<tr>
<th>Day Workers (N= 6)</th>
<th>Night Workers (N= 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Sleep Duration (Time in Bed)</td>
<td>431.3</td>
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<tr>
<td>Wake After Sleep Onset</td>
<td>47.1</td>
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<tr>
<td>Total Sleep Time</td>
<td>389.3</td>
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<tr>
<td>Sleep Percentage</td>
<td>89.8</td>
</tr>
<tr>
<td>Fragmentation</td>
<td>17.7.7</td>
</tr>
</tbody>
</table>