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Calculating the Dim Light Melatonin Onset: The Impact of Threshold and Sampling Rate

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Abstract

The dim light melatonin onset (DLMO) is the most reliable circadian phase marker in humans, but the cost of assaying samples is relatively high. Therefore, the authors examined differences between DLMOs calculated from hourly versus half-hourly sampling and differences between DLMOs calculated with two recommended thresholds (a fixed threshold of 3 pg/mL and a variable "3k" threshold equal to the mean plus two standard deviations of the first three low daytime points). The authors calculated these DLMOs from salivary dim light melatonin profiles collected from 122 individuals (64 women) at baseline. DLMOs derived from hourly sampling occurred on average only 6–8 min earlier than the DLMOs derived from half-hourly saliva sampling, and they were highly correlated with each other ($r \geq 0.89$, $p < .001$). However, in up to 19% of cases the DLMO derived from hourly sampling was >30 min from the DLMO derived from half-hourly sampling. The 3 pg/mL threshold produced significantly less variable DLMOs than the 3k threshold. However, the 3k threshold was significantly lower than the 3 pg/mL threshold ($p < .001$). The DLMOs calculated with the 3k method were significantly earlier (by 22–24 min) than the DLMOs calculated with the 3 pg/mL threshold, regardless of sampling rate. These results suggest that in large research studies and clinical settings, the more affordable and practical option of hourly sampling is adequate for a reasonable estimate of circadian phase. Although the 3 pg/mL fixed threshold is less variable than the 3k threshold, it produces estimates of the DLMO that are further from the initial rise of melatonin.

Keywords

Circadian; Dim light melatonin onset; Phase; Sleep; Threshold

INTRODUCTION

The most reliable measure of the timing of the central circadian clock in humans is the onset of the evening melatonin production measured in dim light, i.e., dim light melatonin onset, DLMO (Lewy et al., 1999). The DLMO is of particular interest for several reasons. First, the DLMO is believed to accurately represent the timing of the central circadian clock (suprachiasmatic nucleus, SCN), as the secretion of melatonin from the pineal gland is controlled by the SCN. Typically, melatonin levels begin to increase in the 2–3 h before the usual onset of nocturnal sleep, peak in the early morning hours, and decrease to daytime

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levels around usual waking. Second, the melatonin rhythm is less easily masked by environmental factors (apart from light) and, indeed, has been shown to produce more reliable circadian phase markers than the core body temperature rhythm (Benloucif et al., 2005; Klerman et al., 2002). Third, the DLMO is a convenient phase marker to measure, because it can be obtained noninvasively (e.g., not requiring a rectal probe or pill to measure the core body temperature rhythm), from saliva (versus plasma, which can be aversive to some people and is associated with a slight health risk), and can require only a relatively short window of sampling of ~6–8 h (Benloucif et al., 2008). For all of these reasons, the use of the DLMO to assess circadian phase is proving to be a valuable tool for both researchers and clinicians alike.

Nonetheless, one drawback in the use of the DLMO as a circadian phase marker is the expense of the melatonin assay (~$12 per sample). The cost of obtaining a DLMO is important given today's restricted research budgets and the likelihood that the assessment of the DLMO will increase in clinical practice. Indeed, the issue of cost raises a question regarding the impact of sampling rate on the calculation of the DLMO: Is there a significant advantage to half-hourly sampling versus hourly sampling, given the additional cost associated with half-hourly sampling? To directly address this question, we examined if the difference between the timing of the DLMO derived from hourly versus half-hourly sampling was statistically or clinically significant. We used two separate low thresholds to determine the DLMO, both recommended in a recent consensus report (Benloucif et al., 2008). These two thresholds do not require sampling of the entire melatonin profile and so are more likely to be used in both clinical and research settings.

METHODS

Subjects

One hundred and twenty-two individual baseline melatonin profiles were compiled from previous studies (Burgess, 2010; Burgess et al., 2008, 2010; Lee et al., 2006; Revell et al., 2005; Smith & Eastman, 2008; Smith et al., 2008, 2009) and unpublished data. Subjects (58 men, 64 women, mean age ± SD 26.3 ± 6.0 yrs, mean body mass index ± SD 23.9 ± 3.0 kg/m^2) were free of prescription medication, except 19 women who used hormonal contraception. All subjects were nonsmokers, in good health, and not extreme morning or evening types as assessed by the Owl-Lark questionnaire (Horne & Östberg, 1976). All subjects passed a urine drug screen for common drugs of abuse at the start of the study. Subjects reported low habitual caffeine (≤300 mg daily) and alcohol (≤2 drinks daily) use and no medical, psychiatric, or sleep disorders based on self-reported screening questionnaires: Minnesota Multiphasic Personality Inventory-2 (Butcher et al., 1989), Beck Depression Inventory (Beck et al., 1961), Pittsburgh Sleep Quality Index (Buysse et al., 1989), and part of a general health questionnaire (Tasto et al., 1978). Subjects gave written informed consent prior to their participation. All studies were approved by the Rush University Medical Center Institutional Review Board and conformed to the standards of this journal (Portaluppi et al., 2010).

Baseline Sleep At Home

All subjects adhered to a regular sleep schedule at home, with fixed bedtimes and wake times, which was between 8 and 9 h long, for 6, 7, 10, or 14 days prior to the baseline circadian phase assessment. Sleep schedules were assigned to match subject's habitual sleep times recorded in the week before the baseline period at home. During the assigned sleep episode, subjects were instructed to remain in bed in the dark even if they could not sleep. Subjects were not allowed to listen to music, talk, read, or watch TV during this time. Subjects wore a wrist actigraphy monitor (Actiwatch-L; Mini-Mitter, Bend, OR, USA),

completed daily sleep logs, and called a voicemail system just before going to bed and after waking each day. Subjects came to the laboratory every 2 to 3 days, and the actigraphy data were downloaded and checked with the logs and voicemails to ensure subjects adhered to the assigned sleep schedule.

Phase Assessment

After their baseline sleep at home, subjects completed a circadian phase assessment in the laboratory to measure their endogenous melatonin profiles. Saliva samples were collected in dim light (<5 lux at subjects' angle of gaze) every 30 min using Salivettes (Sarstedt, Newton, NC, USA). Saliva collection began 6 h before each subject's assigned bedtime and continued for at least 16 h. Alcohol and nonsteriodal anti-inflammatory drugs (NSAIDs) were not permitted for at least 20 h prior to the start of the session. Subjects were breathalyzed upon arrival to ensure blood alcohol concentration was zero. Caffeine was not permitted for at least 6 h before the start of the session. Subjects remained awake and seated in recliners for the duration of the session. Brief restroom (<5 lux) breaks were allowed, but subjects were required to remain seated in the 10 min prior to each sample. Drinks and small snacks between samples were permitted, but subjects were not allowed to eat or drink anything 10 min prior to each sample. If food was consumed, subjects were required to brush their teeth with water while seated; if only liquid was consumed, they were required to rinse with water 10 min prior to each sample. Toothpaste, mouthwash, and edibles containing red food coloring or caffeine were prohibited during the session. Samples were centrifuged and immediately frozen after collection. The frozen samples were later packed in dry ice and shipped to Pharmasan Labs (Osceola, WI, USA) and radioimmunoassayed for melatonin. The sensitivity of the assay was 0.7 pg/mL; the intra- and interassay variability were 12.1% and 13.2%, respectively.

Data Analysis

An hourly sampling rate was derived from the half-hourly profiles by removing every other sample beginning by removing the sample collected 5.5 h before assigned bedtime. For each melatonin profile (derived from half-hourly points or hourly points), we derived two DLMOs using two separate thresholds recommended in a recent consensus report (Benloucif et al., 2008). The first threshold ("3k") was calculated by taking the average of the first three points (which were low daytime levels) plus two standard deviations of these three points (Voultsios et al., 1997; from Dr. David Kennaway's laboratory). Thirteen melatonin profiles were not included in the final data set (n = 122), because they did not have the six low daytime points required for the calculation of the 3k method on the hourly melatonin profiles. The second threshold was a fixed threshold of 3 pg/mL. Three melatonin profiles were not included in the final data set, because melatonin levels failed to exceed 3 pg/mL. For both thresholds, the DLMO was defined as the time that the melatonin levels hit and exceeded the threshold for at least 2 h. The time the melatonin levels crossed the threshold was determined by linear interpolation between the points immediately below and above the threshold. We compared DLMOs with two-tailed paired *t* tests and Pearson correlations.

RESULTS

The 3k DLMO determined from hourly sampling was earlier than the 3k DLMO determined from half-hourly sampling (mean ± SD: 21:18 h ± 51 min vs. 21:26 h ± 56 min, respectively; paired *t* test, $p = .001$; mean difference 8 ± 25 min, difference ranged from hourly 3k DLMO 2 h before to 1.1 h after half-hourly 3k DLMO). Similarly, the 3 pg/mL DLMO determined from hourly sampling was earlier than the 3 pg/mL DLMO determined from half-hourly sampling (mean ± SD: 21:42 h ± 63 min vs. 21:48 h ± 61 min, respectively; paired *t* test, $p < .001$; mean difference 6 ± 11 min, difference ranged from

hourly 3 pg/mL DLMO 1.1 h before to 0.3 h after the half-hourly 3 pg/mL DLMO). As shown in Figure 1 (top panels), there were high correlations between DLMOs derived from hourly versus half-hourly sampling with both thresholds. Nonetheless, the 3k DLMO derived from hourly samples was more than 30 min from the 3k DLMO derived from half-hourly samples in 18.9% of subjects, and more than 1 h different in 5.7% of subjects. The 3 pg/mL DLMOs derived from hourly samples was more than 30 min from the 3 pg/mL DLMO derived from half-hourly samples in 2.5% of subjects, and more than 1 h different in 0.8% of subjects. Figure 1 (bottom panels) shows an example of one subject with at least a 1-h difference between DLMOs derived from half-hourly versus hourly sampling for each threshold.

Overall, the 3k threshold produced significantly more variable DLMOs (equality of variance *F* test, $p < .001$), as the threshold changed when different points were used to calculate the threshold, versus the fixed 3 pg/mL threshold. The 3k DLMOs were on average earlier than the 3 pg/mL DLMOs, regardless of sampling rate (by 22–24 min, both $p < .001$). For the half-hour sampling rate, the 3k DLMO was more than 30 min different from the 3 pg/mL DLMO in 35% of subjects and more than 1 h different in 11% of subjects. With both sampling rates, the 3k threshold (on average 2.3 pg/mL) was significantly lower than the 3 pg/mL threshold (paired *t* tests, $p < .001$). To examine whether the 3k or 3 pg/mL threshold determines the DLMO as a point closer to the initial rise or physiological onset of melatonin secretion, we removed the *y*-axis values on every half-hourly melatonin profile and plotted (but did not label) both the 3k and 3 pg/mL thresholds. We then asked a colleague (S.J.C.; see Acknowledgments) to blindly rate which threshold was closer to the visually identified initial rise in melatonin—where melatonin first rose above baseline levels. She identified the 3k as closer to the physiological onset in 76% of cases (chi-square test of observed vs. random distribution, $p < .001$).

DISCUSSION

This communication examines two related but separate questions. First, given the cost associated with melatonin assays, we examined if estimates of the DLMO would significantly differ if samples were taken every hour, as opposed to every half hour. Second, we determined if the use of two low, but different, thresholds led to any systematic variation in the timing of the calculated DLMO. In response to the first question, the results indicate that when funds are limited, hourly sampling may be sufficient to obtain a reasonably precise estimate of circadian phase. The resulting DLMOs from the two sampling rates were statistically different, but this difference was on average only 6–8 min. The DLMOs from the two sampling rates were also highly correlated with each other, and in only 5% or less of cases did hourly sampling lead to a >1-h (no more than 2-h) difference in the estimation of the DLMO. The level of precision in detecting the DLMO needed for optimal clinical treatment of circadian rhythm sleep disorders remains a matter of debate, but is unlikely to be less than 30 min. Therefore, unless increased resolution in estimating the DLMO is required, hourly sampling may be adequate for studies of large populations or in clinical settings.

The two low thresholds we studied here were recommended by a recent consensus report (Benloucif et al., 2008), specifically because they do not require sampling of the entire melatonin profile. Again, as practicality and cost are important issues in not only research but also clinical practice, the authors of the consensus report suggested every article reporting the DLMO use at least one of these two low thresholds. In this way, it was felt the field could move towards using standardized method(s) for calculating the DLMO, thereby increasing the comparison of data between different research and clinical laboratories. Our results indicate that a systematic difference between these low thresholds does exist. We

found that the 3k method led to an earlier timing (~20 min) in the calculated DLMO relative to the fixed 3 pg/mL threshold. Indeed, a blind rater found the 3k threshold more closely approximated the time of the initial rise in melatonin than the 3 pg/mL in the majority of cases. Notably, however, the 3k method is more difficult to calculate, and the threshold will vary depending on the values of the low daytime points. Thus, although the 3 pg/mL threshold is easier to apply and yielded less variable DLMOs with a change in sampling rate, the 3k method gives a better estimate of the actual point in time when melatonin levels begin to rise.

Limitations to this report do exist. First, we have only analyzed melatonin profiles from normal healthy young subjects. The application of different sampling rates and different thresholds may vary in patient populations or in populations who may secrete less melatonin, such as the elderly. Our data also do not speak to the impact of sampling rate on the many other thresholds currently in use in the field, including those derived as a percentage of melatonin amplitude or from various methods of curve fitting. Although those thresholds are likely to remain as methods of research laboratories, they are not likely to translate into clinical practice. This is because such threshold methods typically require overnight sampling, which increases patient burden and cost. We hope our current study will help guide researchers and clinicians in their decisions regarding choice of sampling rate and threshold when estimating the time of the DLMO.

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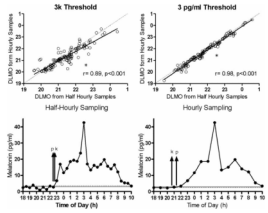

FIGURE 1.

Top panels show correlations (and associated significance level) between DLMOs (time-of-day) derived from hourly and half-hourly sampling rates using 3k (left) and 3 pg/mlmL (right) thresholds. The slope and intercept associated with the 3k threshold were 0.82 and −0.60, respectively. The slope and intercept associated with the 3 pg/mlmL threshold were 1.0 and −0.08, respectively. The asterisk (*) represents a subject with a <1-h difference in the DLMOs determined from hourly and half-hourly sampling; this occurred for both thresholds. The lower panels show the melatonin profiles derived for this subject using half-hourly (left) and hourly (right) sampling rates. The DLMOs calculated with the 3k threshold (threshold shown as dashed line, time of DLMO represented as ↑k), were at 22:36 h for half-hourly sampling and 20:36 h for hourly sampling. The DLMOs calculated with the 3 pg/mlmL threshold (threshold shown as solid line, time of DLMO represented as ↑p), were at 22:32 h for half-hourly sampling and 21:26 h for hourly sampling.