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Changes in Diurnal Salivary Cortisol Levels in Response to an Acute Stressor in Healthy Young Adults[AQ: 1]

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Abstract

BACKGROUND: Knowledge of the diurnal cortisol response to acute stress in healthy individuals can help us better understand the physiological and health effects of chronic stress. **OBJECTIVE:** To compare the diurnal patterns of cortisol secretion of 15 medical students 2 weeks before a major written examination (control phase) and 2 weeks later at the time of the examination (acute stress phase). **DESIGN:** Interrupted time series within-subjects. **RESULTS:** During the acute stress phase, less cortisol was secreted over the course of the day, as demonstrated by a more prolonged and steeper decline in cortisol levels. In addition, higher cortisol levels were present in the evening. Despite these changes in the usual diurnal pattern, overall exposure to cortisol remained the same for both phases. **CONCLUSIONS:** The results of this study suggest that specific adaptations to the diurnal pattern of cortisol are made in the face of acute stress, important information for understanding cortisol regulation in health and illness.

Keywords

physiological stress, medical students, circadian rhythm, acute stress disorder

Since the turn of the 20th century, excess stress has been associated with alterations in health (Johannisson, 2006). Now that we are well into the 21st century, we know that cortisol levels are affected by excess stress and may be a mechanism for developing stress-related health problems. Cortisol levels fluctuate during the waking hours in a typical pattern. Plotted over time, they form two curves: a 30-minute steep upward curve immediately prior to and just after awakening and then a downward curve that lasts the rest of the day, with the steepest decline occurring within the first 3 hours (Buckingham, 2006; Edwards, Clow, Evans, & Huckelbridge, 2001). Under chronic stress, variations in this diurnal pattern have been observed. Probably the most frequently observed variation is a flattening of either diurnal curve. Flattened diurnal curves have been associated with chronic stressors such as economic hardship (Ranjit, Young, & Kaplan, 2005) and parenting a child with cancer (Miller, Cohen, & Ritchey, 2002). Flattened curves are also found in a wide variety of health problems that may be stress related, including fatigue related to breast cancer (Bower et al., 2005), chronic fatigue syndrome (Nater et al., 2008), central obesity (Lasikiewicz, Hendrickx, Talbot, & Dye, 2008), and coronary calcification (Matthews, Schwartz, Cohen, & Seeman, 2006). Despite these observations, there has been little study of the effect of acute stress on the pattern of diurnal cortisol secretion in healthy persons. Knowledge of healthy physiological responses to acute stress is important for nurses whose practices include mental health promotion and counseling on stress reduction and stress management.

Background Literature

Diurnal cortisol secretion is regulated by two interactive biological systems: the circadian "clock" system and the stress system (Nader, Chrousos, & Kino, 2010). The circadian clock system synchronizes internal basal physiological processes to the day and night cycle (Nader et al., 2010). The stress system facilitates the behavioral and physiological adaptations necessary to survive actual and threatened adverse events (i.e., stressors; Chrousos, 2009;

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Rodrigues, LeDoux, & Sapolsky, 2009). Cortisol is but one of numerous bio-substances regulated by the circadian clock and stress biological systems. The hypothalamic–pituitary–adrenal (HPA) axis functions to regulate specifically the production of cortisol. Stimulation of a specialized location in the hypothalamus initiates the physiological cascade that results in cortisol secretion from the adrenal cortex into the blood stream. Stress-related cortisol elevations generally return to basal levels after the stressor has ceased, because of a negative feedback system within the HPA axis that is mediated by glucocorticoid receptors, particularly in the hippocampal region of the brain (Yu, Holsboer, & Almeida, 2008). The circadian clock and stress systems mutually regulate each other. There is growing evidence that dysregulation of either system due to chronic stress can eventually lead to serious health problems (Nader et al., 2010).

The effect of acute stress on cortisol secretion in humans can be studied in either laboratory or natural settings. Although robust in standardization and control, laboratory studies lack ecological validity (Kudielka, Hellhammer, & Wüst, 2009). Natural settings can provide ecological validity, but the trade-off is reduced standardization and control. Nonetheless, there is a growing body of research on the effects of stress on cortisol secretion that focuses on students, workers, athletes, and military personnel, among others, outside the laboratory setting. To illustrate, the authors of a meta-analysis on cortisol response to acute or chronic stress in healthy adults in natural settings identified 140 eligible studies published between 1978 and March 2007 (Michaud, Matheson, Kelly, & Anisman, 2008). The data from these studies demonstrated a moderate effect size (0.61) for overall cortisol response (Michaud et al., 2008). This meta-analysis suggests that it is both feasible and productive to study the effect of acute stress on cortisol secretion in natural settings.

Measurement of the effect of acute stress on diurnal cortisol secretion is usually accomplished by measuring cortisol during a control phase and then at the time of the acute stressor, the acute stress phase. To measure the pattern of diurnal cortisol secretion, samples from blood or saliva need to be taken two or more times over the course of the day. Despite the large number of studies included in the Michaud et al. (2008) meta-analysis, the effect of acute stress on diurnal cortisol secretion was the focus of only three studies (Evens, Bristow, Hucklebridge, Clow, & Pang, 1994; Malarkey, Pearl, Demers, Kiecolt-Glaser, & Glaser, 1995; Vedhara, Hyde, Gilchrist, Tytherleigh, & Plummer, 2000). The acute stressor was the same for all three studies: an academic examination. A search for recent comparable studies did not yield any additional studies of the affect of an academic examination on diurnal cortisol secretion.

Evans et al. (1994) and Malarkey et al. (1995) compared diurnal cortisol secretion during the control and acute stress phases by aggregating their multiple diurnal cortisol measures into a daily mean. Evens et al. found increased cortisol levels at the time of the examination, whereas Malarkey et al. found no change but did find decreased levels of cortisol at an additional measurement point 2 weeks after the exam. In contrast, Vedhara et al. (2000) compared diurnal cortisol secretion during the control and acute stress phases using repeated measures analysis of variance (ANOVA). Unexpectedly, they encountered lower levels of cortisol at the time of the examination when compared with the control phase. One explanation is that bias may have been introduced into the Vedhara et al. study from (a) sampling a heterogeneous group of students and (b) a lack of control on the actual examinations taken by the students.

The statistical methods used by Evens et al. (1994), Malarkey et al. (1995), and Vedhara et al. (2000) placed additional limitations on their studies. First, aggregation of diurnal cortisol measures into daily means loses information on the strength of the cortisol response and its transformation over time (Fekedulegn et al., 2007). One alternative is area under the curve (AUC) calculations, which convert multivariate data into univariate space (Fekedulegn et al., 2007). However, if cortisol data are collected over multiple days—which increases reliability in studies conducted in natural settings (Hruschka, Kohrt, & Worthman, 2005), information on within-subject variability can be lost if the means for several days are used for each time point when calculating AUC. The repeated measures ANOVA approach used by Vedhara et al. (2000) is a viable alternative, but this approach is limited by the need for balanced data without any missing or inconsistently timed observations (Wallace & Green, 2002). A better alternative for studies conducted in natural settings is the repeated measures mixed linear model approach, which does not require balanced data. In addition, repeated measures mixed linear model analysis can provide information on diurnal curve characteristics because this statistical method is based on the hierarchical linear model/random coefficient approach, as well as the repeated measures ANOVA approach (Wallace & Green, 2002).

Purpose

The purpose of this study was to compare the diurnal patterns of cortisol secretion of young, healthy medical students 2 weeks before a major written examination (control phase) and 2 weeks later at the time of the examination (acute stress phase). Our primary specific aim was to determine differences between the control and the acute stress phases in (a) AUC cortisol and (b) characteristics

of the diurnal cortisol decline as determined by repeated measures linear mixed model analysis. The secondary specific aims were to (a) determine the level of anxiety and depression in the students during the control and the acute stress phases and (b) pilot a daily diary designed for collecting data on self-reported saliva collection times and potential confounding variables.

The medical school examination model of acute stress typically elicits both psychological and biological markers of stress, including increases in anxiety (Harris & Martin, 1994; Herbert, Moore, de la Riva, & Watts, 1986; Shepard, al'Absi, Whitsett, Passey, & Lovallo, 2000), perceived stress (Malarkey et al., 1995), adrenocorticotropic hormone levels (Malarky et al., 1995), basal cortisol levels (Harris & Martin, 1994; Jones, Copolova, & Outch, 1986; Shepard et al., 2000), noradrenaline levels (Herbert et al., 1986), and blood pressure and heart rate (Harris & Martin, 1994; Shepard et al., 2000). A major medical school examination is an example of a "motivated performance task," which was defined by Dickerson and Kemeny (2004) as a task in which an important goal is threatened by the potential for poor performance and uncontrollability. The inherent need to preserve the social self may help explain why motivated performance tasks elicit 3 times the cortisol response as other acute stressors in the laboratory setting (Dickerson & Kemeny, 2004).

Method

Study Design

An interrupted time series within-subjects study design was used (Burns & Grove, 2009). Fifteen students collected saliva samples every 2 hours (from 08:00 to 22:00 hours) for a total of 6 days during two phases. The control phase was 2 weeks before the medical exam (Days 1-3, Thursday through Saturday). The acute stress phase was exactly 2 weeks later (Days 4-6, Thursday through Saturday), with the exam administered on Day 6 (Saturday).

The medical school examination covered a large amount of material provided in a lecture format over the previous 3 weeks and took 3 to 4 hours to complete. Anecdotally, the stress of these exams was high because scoring at the bottom of the class was undesirable, regardless of the percentage correct achieved. To document the stressful nature of the exam psychologically, we measured anxiety at the beginning of both the control and acute stress phases. We also measured depression as a potential confounding variable, because (a) depression has been associated with changes in diurnal cortisol secretion (Peeters, Nicolson, & Berkhof, 2004) and (b) medical school may increase risk for depression (Rosal et al., 1997, Stecker, 2004).

Sample

The study was approved by the Institutional Review Board at the University of Nebraska Medical Center and the College of Medicine, University of Nebraska Medical Center. A convenience sample of 15 first-year medical students (10 males and 5 females) was recruited by class and e-mail announcements. Each student signed a written informed consent before data collection. The students' mean age was 23.4 years (SD = 1.06 years, range 22-26 years). Ineligibility criteria included tobacco use, current psychotropic medication use, alcohol or substance abuse in the past 6 months, oral or pulmonary steroid use in the past 3 months, irregular menses, and major medical problems.

Paper and Pencil Measures

Anxiety. Anxiety was measured by the State–Trait Anxiety Inventory for Adults (STAI; Spielberger, Gorusch, Lushene, Vagg, & Jacobs, 1983). The STAI consists of two 20-item scales, designed to clearly differentiate between the temporary condition of state anxiety and the more general and long-standing quality of trait anxiety. Scores for each subscale range from 20 to 80 with higher scores indicating higher levels of anxiety. Evidence of internal consistency for state (.88-.93) and trait (.92-.94) anxiety, test–retest reliability for trait anxiety (.73-.86), and content and construct validity have been demonstrated (Groth-Marnat, 2009).

Depression. The Beck Depression Inventory–II (BDI-II; Beck, Steer, & Brown, 1996) was used to measure depression. The BDI-II is based on diagnostic criteria for depressive disorders. Twenty-one groups of four-item statements are rated according to the way the respondent felt in the past week. Scores can range from 0 to 63. Higher scores indicate more severe depression symptomatology. Internal consistency has ranged from .89 to .94. One-week test–retest reliability was found to be .93. The BDI-II has been shown to discriminate primarily anxiety disorders from primarily depressive disorders (Groth-Marnat, 2009).

Daily diary. A daily diary was developed by the first author for students to write down the times they woke-up, went to bed, ate, exercised, and experienced acute stress. In addition, students recorded in the diary the times they actually collected their saliva samples as a measure of compliance with the study protocol.

Procedure

An individual appointment was made for interested students to go over study eligibility, discuss and sign the informed consent (if eligible and interested), and chose a four-digit personal identification number (PIN). A group

meeting for consented students was scheduled over the students' lunch break the day before data collection began (Wednesday) for the control phase. During the meeting, the students received verbal and written instructions for collecting saliva samples. The control phase daily diaries were distributed and explained. In addition, students filled out the STAI and the BDI-II, and information was gathered on their gender and age.

Specific directions for saliva sampling included (a) collect samples every 2 hours from 8 a.m. to 10 p.m.; (b) before collecting the sample, rinse mouth with water, which may be swallowed; (c) insert the cotton dental roll found in the salivette labeled with correct time and date and chew gently for 1 minute; and (d) place dental roll in the special insert inside the salivette. The students then practiced on a spare salivette the proper technique for obtaining a saliva sample and placing it back into the salivette.

The students were provided with a ziplock bag marked with their PIN and the next day's calendar date and day of the week (Thursday). Inside were eight salivette tubes marked with the same information, along with a specific time for saliva collection, in regular and military time. Each salivette also had a unique identifier number on its end. This identifier number was placed in a database that linked it with the student's PIN and the date and time of day the specimen was to be collected. To facilitate timely saliva sampling, students were each provided a Cadex™medication reminder watch set to beep at the appropriate 2-hour intervals. Bottles of water were also supplied for mouth rinsing before collecting specimens. Students were instructed to bring back their Day 1 specimens 2 days later (Friday), before their 8 a.m. class.

When students returned their Day 1 specimens that Friday, they were asked if they had difficulties or questions. Only two students missed one or more collection times on Day 1. Before they left on Friday, students were provided with a ziplock bag with salivettes for Day 2 (to be used that same day) and a ziplock bag with salivettes for Day 3 (to be used the next day, Saturday), labeled in the same manner as for Day 1. They were instructed to return the bags with their saliva samples on the following Monday before their 8 a.m. class. Students were requested to store each day's bag of salivettes in the freezer at the end of the day until delivery. On Monday, when students returned with their salivettes, they also returned their control phase daily diaries and their Cadex™ watches.

The procedure for the acute stress phase was exactly the same, except for the distribution of the salivettes. Because there were no classes scheduled for that Friday (Day 5), students were provided at the Wednesday group meeting with three ziplock bags containing salivettes for all 3 days of the acute stress phase (Days 4, 5, and 6). They were to return all of their specimens the following Monday. Some students forgot to return them that Monday but kept them frozen until their later return. Saliva specimens were stored at −20°C until assayed at the Endocrine Bioservices Laboratory at the University of Nebraska at Omaha. Students were provided a small monetary compensation at the end of both the control and acute stress phases.

Cortisol Determination

An enzyme-linked immunoassay specific for saliva was used for the measurement of cortisol levels. Additional details of the assay methodology are found in Smith and French (1997), and the assay has been validated for salivary cortisol in human participants (Elverson, Wilson, Herzog, & French, in press; Minton, Hertzog, Barron, French, & Reiter-Palmon, 2009. Intra-assay coefficients of variation were 7.67% and 3.83% for a high-concentration and low-concentration pool, respectively. Interassay coefficients of variation were 21.80% and 11.16% for a high-concentration and low-concentration pool, respectively.

Data Analysis

The Statistical Package for Social Sciences for Windows was used for statistical analysis. Level of significance for all data analyses was set at .05. Scores for the STAI and the BDI-II were calculated as the sum of values endorsed for each item of the scale. Descriptive data obtained from the daily diaries included (a) whether a recording was made for each requested sample and the number of minutes in which each sample was collected late or early and (b) the daily times recorded for waking up, bedtime, meals, exercise times, and non-exam-related stress. The two-tailed paired-samples *t* test was used for all bivariate analyses.

To prepare the cortisol data for calculation of AUC, the phase mean for each time point of saliva collection was determined. In the one case in which one student's cortisol samples were missing for all 3 days at one particular time point, the mean of the time points immediately before and after the missing time point was used. The trapezoid method was used to determine AUC for each phase (Pruessner, Kirschbaum, Meinlschmidt, & Hellhammer, 2003).

Two AUC measures were calculated. The first was AUC with respect to ground (AUC_{ground}). The bottom parameter for AUC_{ground} consisted of a straight line fixed at zero (Pruessner et al., 2003). As a measure, AUC_{ground} represents total cortisol output (Fekedulegn et al., 2007). The second AUC measurement was AUC with respect to the lowest cortisol measurement of the day, labeled AUC_{low}. The bottom parameter for AUC_{low} was the straight line fixed at the 22:00 h cortisol measurement. By holding the lowest cortisol level constant, the calculation of AUC_{low} provided a measure of the steepness of the decline in cortisol levels over the course of the day (Fekedulegn et al., 2007).

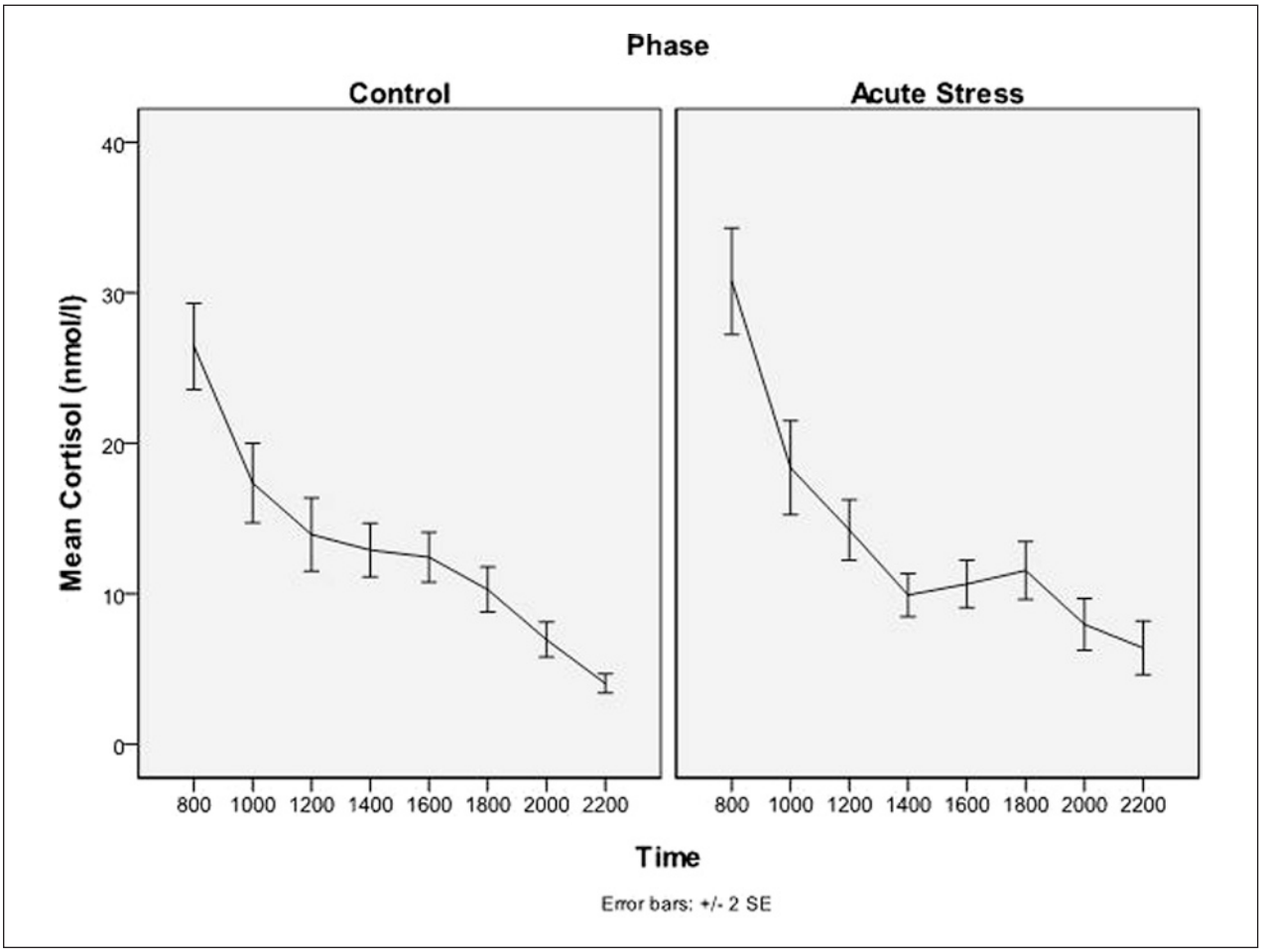

Figure 1. Mean (±standard error of the mean) cortisol values by time and phase ($N = 15$)

Characteristics of the diurnal cortisol decline were examined using repeated measures linear mixed model analysis. As stated above, repeated measures linear mixed model analysis is particularly well suited for analyzing diurnal patterns of cortisol secretion because no data are eliminated from analysis, and within-individual variation is analyzed at the same time as between-individual variation (Hruschka et al., 2005).

Results

Of a potential total of 720 saliva samples, 645 (89.6%) were collected and assayed, leaving a total of 75 (10.4%) missing cortisol values. Eight students collected all 48 saliva samples. Of the seven students who did not collect all of the samples, one female student dropped out of the study after Day 2, and another female student dropped out after Day 3, accounting for 56 of the missing samples. Of the remaining 19 missing samples (distributed over five students), 12 were from the control phase and 7 were from the acute stress phase. Mean cortisol values for each time point during the control and acute stress phases are portrayed in Figure 1 ($N = 15$). The expected decline in cortisol values over the course of the day is clearly visible in each phase.

AUC Cortisol by Phase

The two students who dropped out during the control phase were eliminated from all bivariate comparisons by phase. The mean AUC_{ground} and AUC_{low} cortisol values for each phase are compared in Figure 2 ($N = 13$). Mean AUC_{ground} cortisol was not significantly different by phase ($t = 0.139$, $p = .891$). However, mean AUC_{low} cortisol fell 25% from the control phase to the acute stress phase ($t = 2.341$, $p = .037$). This difference was likely in part because of the 57% higher mean cortisol values at 22:00 hours during the acute stress phase ($t = 2.408$, $p = .033$). Morning mean cortisol levels were also higher (24%) at 08:00 hours for the acute stress stage, but

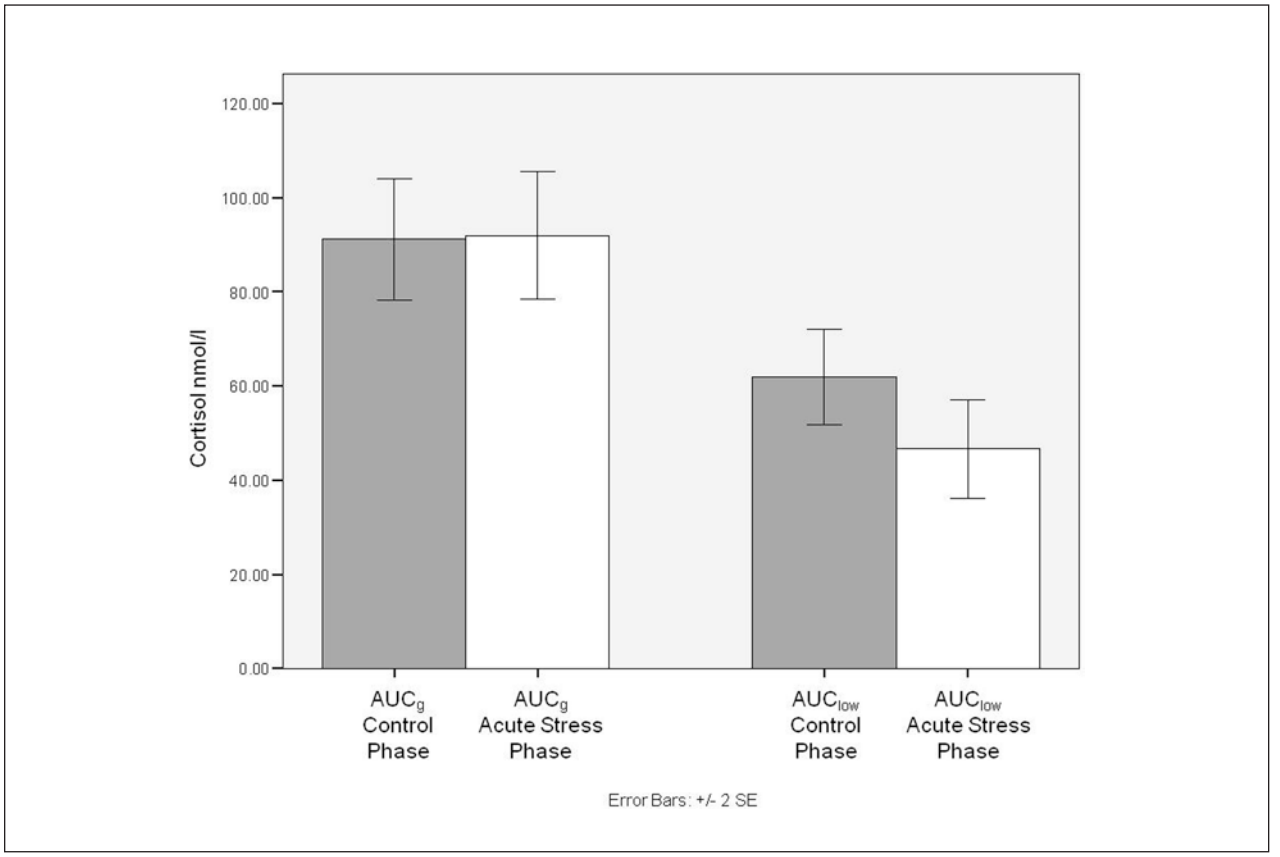

Figure 2. Comparison of control phase and acute stress phase AUC_{ground} (AUC_g) and AUC_{low} values (N = 13)
Note. AUC = area under the curve. Values are not true values of AUC but rather a linear transformation of AUC_{ground} and AUC_{low} (Pruessner et al., 2003). AUC_{low} was significantly ($p \leq .05$) lower in the acute stress phase than in the control phase.

this difference did not achieve significance (t = 1.739, p = .108).

To rule out that a later wake-up time during the acute stress phase delayed students from arriving at their 24-hour cortisol low at 22:00 hours, mean wake-up times were compared by phase. The mean time students woke up was virtually the same for both the control phase (07:02 hours, SD = 32 minutes) and the acute stress phase (06:57 hours, SD = 49 minutes). Another explanation could have been sleep deprivation, which has been associated with higher evening cortisol levels in healthy populations (Omisade, Buxton, & Rusak, 2010; Spiegel, Leproult, & Van Cauter, 1999). Sleep duration was calculated by subtracting the bedtimes of the first 2 days from the next morning's wake-up times, allowing for two measurements per phase (N = 12). The mean duration of sleep for the control phase was 6 hours and 56 minutes (SD = 1 hour and 19 minutes), and for the acute stress phase, it was 7 hours and 5 minutes (SD = 1 hour and 3 minutes), a nonsignificant difference (t = 0.735, p = .478).

Characteristics of the Diurnal Cortisol Decline by Phase

The results of the repeated measures linear mixed model analysis (N = 15) are presented in Table 1. The only significant main effect was for time, which was expected given the natural circadian rhythm of cortisol secretion. However, there was a significant phase × time interaction. Therefore, the significance of the changes in cortisol levels for each time interval was examined for each phase. Significant changes were found in both the control and acute stress phases during the two morning time intervals: 08:00 to 10:00 hours and 10:00 to 12:00 hours. During the acute stress phase, cortisol levels in the 12:00 to 14:00 hours time period continued to significantly drop (p = .001), whereas they leveled off in the control phase. From 14:00 to 16:00 hours, there were no significant changes in cortisol levels in either phase. But there was a significant drop during the control phase from 16:00 to 18:00 hours (p = .042) that was not shared by the acute stress phase.

Table 1. Repeated Measures Linear Mixed Model Analysis Results by Phase, Day, and Time ($N = 15$)

Source	Numerator *df*	Denominator *df*	*F*	Significance
Phase	1	398	0.640	.424
Day	2	253	0.118	.888
Time	7	102	103.925	.000
Phase × day	2	253	0.678	.509
Phase × time	7	102	2.359	.028
Day × time	14	67	0.461	.946
Phase × day × time	14	67	0.809	.657

Note. *df* = degrees of freedom.

Finally, both phases dropped significantly during the 18:00 to 20:00 hours and 20:00 to 22:00 hours time periods.

Anxiety and Depression Results

Mean values for the STAI's Trait Anxiety Scale ($N = 13$) were about the same for the control phase (27.62; $SD = 6.16$) as for the acute stress phase (27.31; $SD = 6.51$). However, mean values for the State Anxiety Scale ($N = 13$) tended to be higher in the acute stress phase (29.69; $SD = 11.46$) than in the control phase (24.54; $SD = 4.52$; $t = 2.076$, $p = .060$). The mean BDI-II score for the control phase was 2.77 ($SD = 3.00$; range 0-9), and the acute stress phase mean was 3.69 ($SD = 4.64$; range 0-13). Because a score of 14 is the cutoff score for mild depression in the general population (Younkers & Sampson, 2008), depression was apparently not an issue for the participants of this study.

Daily Diary Results

Across all of the diaries, except for one time during the control phase and nine times during the acute stress phase, the students faithfully recorded every time they actually collected their saliva samples. They appeared to be quite compliant with the timing of their saliva sampling. For each day, the following describes the number of samples that were obtained more than 10 minutes late or early by day: Day 1, 7 samples (6.1% of saliva samples collected that day); Day 2, 19 samples (16.5%); Day 3, 11 samples (10%); Day 4, 7 samples (6.8%); Day 5, 15 samples (14.6%); and Day 6, 14 samples (13.9%). Only seven saliva samples (1.1%) were greater than 30 minutes off target, a small enough percentage that their exclusion from data analysis would not make a difference in the study results.

Meal times were correctly filled out and yielded little further information. Of the students who completed both phases, breakfast was consumed 92.3% of the time; lunch, 94.9%; and dinner, 96.3%. Snacks were consumed at various times of the day, and reported by most students. Four students reported a non-exam-related stress episode during the control phase, and two students reported a non-exam-related stress episode during the acute stress phase. Nine students reported exercising at least once during the control phase, and eight students reported exercising at least once during the acute stress phase.

Discussion

The effect of acute stress on the diurnal pattern of cortisol secretion was studied in a natural setting. Young, healthy medical students served as their own controls; they sampled their saliva for cortisol every 2 hours eight times a day during a 3-day control phase and then exactly 2 weeks later during a 5-day acute stress phase. The acute stressor was an important medical school examination, which was timed to occur on the last day of the acute stress phase. In this natural setting study, certain key aspects of the participants and their lives were homogenous, including their youthful age and health, school and examination schedule, and low depression and trait anxiety scores. In addition, mean wake-up times and hours of sleep were stable by phase.

Two statistical approaches were used to analyze differences in the diurnal pattern of cortisol secretion during the control and acute stress phases: AUC and repeated measures linear mixed model analysis. The AUC calculations provided information on total cortisol output (AUC_{ground}) and the steepness of the diurnal decline (AUG_{low}). Using this information, the intensity of the HPA axis response (AUC_{ground}) and its sensitivity to the acute stressor (AUG_{low}) can be deduced by comparing the two phases (Fekedulegn et al., 2007). Comparison of mean AUC_{ground} cortisol during the acute stress phase and control phases demonstrated no differences, suggesting that total cortisol exposure remained unchanged during the acute stress phase. However, AUC_{low} dropped a quarter of its value during the acute stress phase. These findings imply that the steepness of

the diurnal curve's decline was greater for the acute stress phase than for the control phase, despite higher mean cortisol values at the end of the day.

Further information was gained on differences in the characteristics of the diurnal curves through the repeated measures linear mixed model analysis. The significant phase × time interaction indicated that the characteristics of the diurnal curves of the control and acute stress phases were different. The typical pattern of a steep decline in cortisol levels for 3 hours after the initial ½ hour of awakening was reflected in the control phase, but in the acute stress phase the steep decline continued into the early afternoon. Therefore, the morning quick decline in cortisol secretion during the acute stress phase was more prolonged compared with the control phase. Taken together, the AUC_{low} calculations and repeated measures linear mixed analysis suggest that against a background of higher evening levels of cortisol, the HPA axis secreted less cortisol over the course of the day during the acute stress phase than during the control phase. Yet these homeostatic adjustments did not result in increased diurnal cortisol exposure overall, as indicated by the AUC_{ground} cortisol results.

It is notable that diurnal cortisol levels were not markedly different on the day of the medical school examination, when compared with the other days of the acute stress phase. A plausible explanation is that the students were in a state of anticipatory stress in the days leading to the examination, which was captured by the control and acute stress phase differences in (a) AUG_{low} and (b) characteristics of the diurnal cortisol decline. Adjustments to basal cortisol levels by the HPA axis in response to an anticipated stressor may play a role in survival because basal cortisol levels are initially involved in the stress response (Jankord & Herman, 2008; Sapolsky, Romero, & Munck, 2000). The phenomenon of cortisol levels rising minutes or hours in advance of a known acute stressor has been noted in laboratory settings (Kirschbaum, Pirke, & Hellhammer, 1993) and in natural settings (Salvador, Suay, Gonzalez-Bono, & Serrano, 2003), but there has been scant documentation, outside of this study, that the diurnal pattern of cortisol secretion may also change during the days before a known acute stressor.

The anticipated stress explanation for the changes between the control and acute stress phases in diurnal cortisol secretion is partially supported by the state anxiety results. The students took the STAI on the day before data collection for the acute stress phase (the Wednesday before their Saturday examination), and the 6.4% increase in state anxiety from the control phase approached significance ($p = .060$). Nonetheless, this increase is relatively small. We measured state anxiety to document the psychological stress of the examination, but for this purpose it would have been better to have measured this variable on the day of the examination. Interestingly, it was recently noted that measures of self-reported stress appear to explain little of the variance in physiological stress responses (Hellhammer, Wüst, & Kudielka, 2009). Either this approach to documenting stress may need rethinking or better measures of self-reported stress may need to be developed (Hellhammer et al., 2009).

There are two additional alternate explanations for no significant differences in diurnal cortisol patterns on the actual day of the medical school examination. One alternate explanation could be habituation (Lester, Brown, Aycock, Grubbs, & Johnson, 2010). The examination was scheduled during the medical students' second semester of their first year, so they were experienced with this particular type of acute stressor. Habituation may help explain the relatively low state anxiety score changes but would not explain the phase differences in diurnal cortisol secretion. Another explanation could be that attending medical school in itself is a chronic stressor, which would alter the HPA axis response to acute stressors (Cacioppo et al., 2000). However, the students were not yet in clinical rotations, which are the major source of medical school stress (LeBlanc, 2009). In addition, chronic stress is a harbinger for anxiety and depression, of which the students scored low. An additional measurement 2 weeks later during which no examinations were given would have strengthened the evidence that the changes between phases were directly related to the acute stress of the examination.

The students completed the piloted daily diary as directed, demonstrating its value. Overall, the students were remarkably compliant in their adherence to a relatively rigorous protocol for data collection. However, they might not have been as reliable as they appeared to be with regard to collecting their saliva samples on time. In a study of diurnal cortisol in women with fibromyalgia and control participants, the salivettes were equipped with special caps to monitor when they were opened (Broderick, Arnold, Kudielka, & Kirschbaum, 2004). Noncompliance was defined as more than 15 minutes off for awakening samples and more than 60 minutes off for afternoon and evening samples, the latter of which were obtained at 3-hour intervals. The self-reported compliance rate was high (≥92%), but the true compliance rate dropped to 80% in participants with fibromyalgia who were unaware of the special caps and to 62% in unaware control participants. The differences in findings in noncompliant participants were great enough to alter the overall results.

Limitations

Our study has several limitations in addition to self-collected saliva samples. The first limitation is the relatively small sample size ($N = 13$) for the bivariate AUC statistical analyses. Besides affecting the statistical power, the sample

size did not allow for exploring potential moderating factors such as gender. A second limitation is the timing for diurnal cortisol measurement, which was patterned after Ice, Katz-Stein, Himes, and Kane's (2004) study on diurnal cortisol patterns in older adults. Subtle aspects of the diurnal pattern of cortisol secretion may have been obscured by (a) timing cortisol sampling to clock times rather than waking times and (b) not measuring the 30-minute rise in cortisol after awakening (Edwards, Hucklebridge, Clow, & Evans, 2003). A third limitation pertains to all studies of natural acute and chronic stressors outside of laboratory control. Lack of experimental control allows for the introduction of extraneous variables that may influence results but not be reproducible. For example, it was found out anecdotally after the study terminated that some medical students, on a regular or irregular basis, do not attend class. It is unknown what proportion of the participants of this study attended class regularly.

Conclusion

In conclusion, the physiological adjustments to acute stress made by the human organism help maintain homeostasis and promote survival. The results of this study suggest that subtle and specific adjustments in the diurnal pattern of cortisol secretion occur in anticipation of a known acute stressor. This study is important because the human response to acute stress in natural settings, as measured by patterns of diurnal cortisol secretion, has been little studied. The information gained from this study can help acquire a fuller understanding about the trajectory of cortisol changes from acute stress to chronic stress to stress-related health problems, important knowledge for the many nurses who have interest and expertise in mental health promotion and stress counseling. Further research is needed to replicate and contrast these findings with diurnal patterns of cortisol secretion in response to unanticipated acute stress. In addition, it may be beneficial for researchers of diurnal patterns of cortisol secretion in chronically stressed or ill individuals to measure and control for anticipated acute stress to avoid confounding their results. This further research will help extend knowledge on the complex nature of cortisol regulation in health and illness.

Author Roles

Polly A. Hulme contributed to designing the study, collecting the data, conducting the cortisol assays, data entry, statistical analysis, and writing the article. Jeffrey A. French contributed to designing the study, conducting the cortisol assays, and writing the article. He also provided his laboratory for conducting the cortisol assays. Sangeeta Agrawal contributed to the statistical analysis of the study.

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