An Undergraduate Physiology Laboratory Module of Salivary Cortisol Measurement with an Emphasis on Circadian Rhythmicity and Quantitative Analysis

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Abstract

An undergraduate physiology lab module was developed to enhance student understanding of the functions of cortisol hormone, the circadian rhythm associated with cortisol release, proper ELISA technique, and quantitative data analysis methods including linear regression and t-test assessment. The statistical analysis was accomplished using Microsoft Excel. Students measured their cortisol levels in saliva samples collected in late evening and early morning using an ELISA kit. Both subjective and objective assessments showed that the learning objectives were successfully achieved.

** He Liu and Mary Vagula presented a poster on this laboratory module at the 29th HAPS Annual Conference in San Antonio, Texas in May 2015.

Key words: undergraduate education, physiology lab, salivary cortisol, circadian rhythm, sleep

Introduction

The National Research Council recommended that "a new biology curriculum" should include quantitative principles and skills (National Research Council 2003, 2009), which are not commonly included in biology laboratory courses. In addition, many physiology laboratory courses lack components of biochemical methods and activities related to the human endocrine system or circadian rhythms. Taking advantage of the circadian rhythmicity of cortisol, which has a low level in the evening and a high level in the morning (Wehr *et al.* 2001, Kim *et al.* 2015), we developed a lab module for students interested in the health care professions and other biology majors. Students used an ELISA kit to measure their cortisol levels in saliva samples that were collected in late evening and early morning. Microsoft Excel was used to analyze the experimental data. Our goals were to enhance student understanding of human endocrine physiology and circadian rhythms, and to introduce quantitative skills such as linear regression and statistical hypothesis testing to undergraduate students. Both subjective and objective assessments, including a post-lab questionnaire and homework lab report grades, showed that the goals were successfully achieved. The use of student cortisol measurement data and the publication of feedback results were approved by the Institutional Review Board at Gannon University, #15-02-05.

Material and Methods

Materials Needed: Ultrasensitive Cortisol Saliva ELISA Assay Kit (Eagle Biosciences, Nashua, NH) was purchased for this lab. One kit (96 wells) is sufficient for 44 students as well as for a set of wells needed to construct the standard curve. In addition, a microplate reader (Promega, Madison, WI) equipped with a 450nm filter, a vacuum apparatus, micropipettes and tips, and a horizontal shaker, were used for this exercise.

Sample collection: Students were instructed to collect saliva samples at home before and after sleep. After brushing teeth and rinsing mouth thoroughly with water, students waited 10 minutes to accumulate saliva in the floor of the mouth. Saliva samples were collected by tilting the head forward and letting the saliva flow into microcentrifuge tubes. Microcentrifuge tubes of two colors were given to students to avoid mislabeling (blue for night and red for morning). Students kept the samples frozen prior to submission, and instructors froze the samples again prior to the lab. Freeze/thaw cycles do not alter cortisol concentration but will prevent odor from developing in the saliva samples (Kalman & Grahn 2004). For privacy protection, collected samples were taken from students, re-labeled with numbers, and distributed randomly back to the students before measurements were taken.

Cortisol measurement: Cortisol levels were measured following the ELISA kit instruction manual. Saliva samples, the antibody, and Cortisol Horseradish Peroxidase (HRP) conjugate reagent were mixed

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in wells pre-coated with goat anti-rabbit γ-globulin and incubated at room temperature for 60 minutes on a shaker. After the incubation, students used a vacuum apparatus to wash the wells. Then the color development reagent was added to each well and incubated for another 30 minutes before the stop solution was added to stop the color development. The optical density (O.D.) was read using a microplate reader with a 450 nm filter. The plate O.D. data was sent to students by email.

Learning objective assessments: Following the exercise, students completed a lab report with questions based on their knowledge of cortisol physiology and the results/conclusions of the data analysis. In addition to lab reports, students also completed a questionnaire anonymously that provided subjective feedback on this lab (Table 2).

Results

Class activity: Repeated measurements of the standards showed little variation. Therefore, one set of standard measurements was shared across multiple lab sections. The standard measurements can be made by the instructor prior to the lab or with students during the lab. Students were divided into groups of four and each group worked on one strip of eight ELISA wells. The ELISA procedure includes two incubation periods: a 60-minute incubation for antibody binding and a 30-minute incubation for color development. During the first incubation period, students were given a lecture on topics including the endocrine system, circadian rhythms, the ELISA method, linear regression, and hypothesis testing. Students were shown how to construct an ELISA standard curve with linear regression and a paired t-test. During the second incubation period, students were given Microsoft-Excel files to practice computer-based data analysis skills. Pre-recorded demonstration videos were provided to assist students through the practice during the lab and data analysis after the lab.

Student lab results: The log-log model was used in standard curve construction for its simplicity (Plikaytis *et al.* 1991) (Table 1, Figure 1B). In the student data, a 3-fold difference was observed between evening and morning salivary cortisol levels (Figure 1C). The difference was visible during and after color development (Figure 1A).

Assessment: Four multiple-choice questions were included in the student questionnaire to rate the effectiveness of this lab. For each question, students were given choices of "Not at all (none)", "A little bit", "Some", "A lot", and "Definitely a lot". For quantitative analysis, the answers were weighted with a 5-point scale with 1 being "none" and 5 being "definitely a lot". The questions and the distributions of the answers are shown in Table 2 and Figure 2. Student responses were positive for the first two questions in the questionnaire

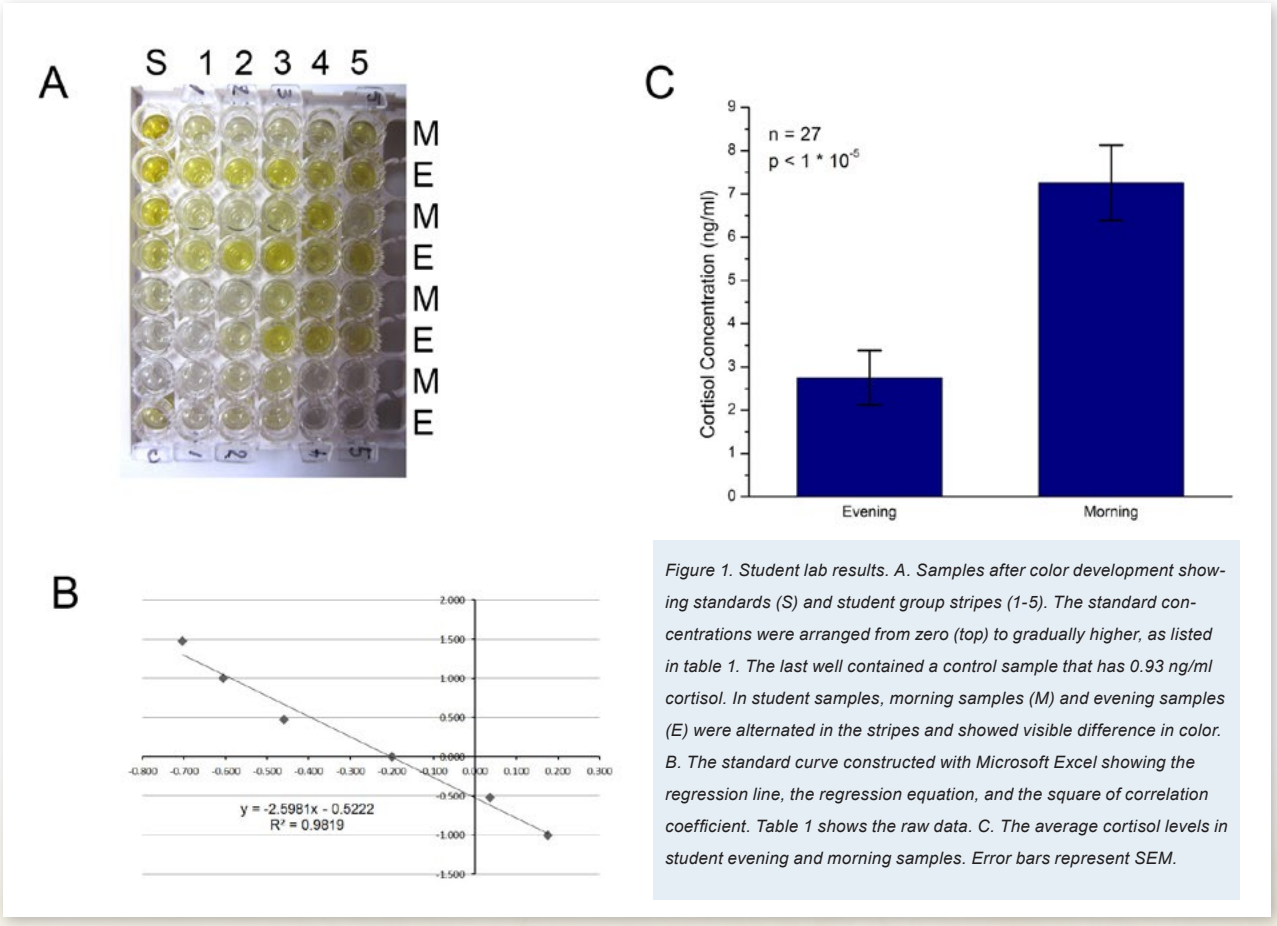

Figure 1. Student lab results. A. Samples after color development showing standards (S) and student group stripes (1-5). The standard concentrations were arranged from zero (top) to gradually higher, as listed in table 1. The last well contained a control sample that has 0.93 ng/ml cortisol. In student samples, morning samples (M) and evening samples (E) were alternated in the stripes and showed visible difference in color. B. The standard curve constructed with Microsoft Excel showing the regression line, the regression equation, and the square of correlation coefficient. Table 1 shows the raw data. C. The average cortisol levels in student evening and morning samples. Error bars represent SEM.

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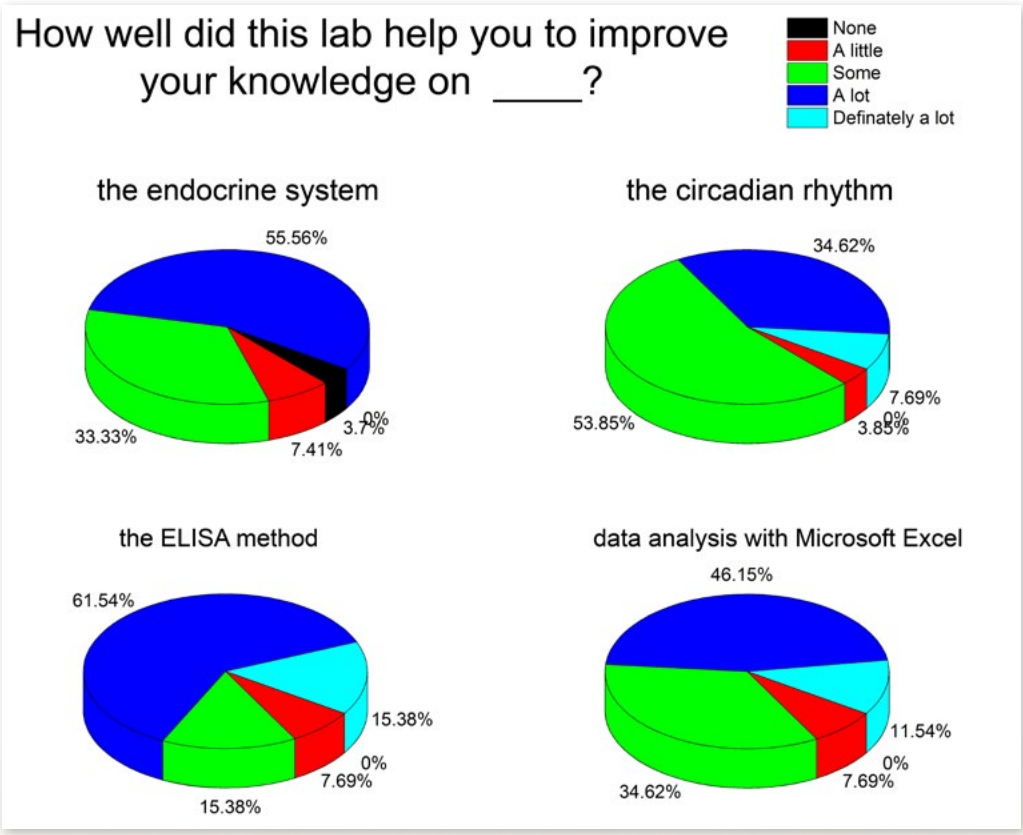
Table 1. Standard Measurements

Standard	OD	Concentration(ng/ml)	log(OD)	log(concentration)
#1	1.832	0		
#2	1.501	0.1	0.176	-1.000
#3	1.086	0.3	0.036	-0.523
#4	0.631	1	-0.200	0.000
#5	0.347	3	-0.459	0.477
#6	0.248	10	-0.606	1.000
#7	0.198	30	-0.704	1.477

Table 2. Student Feedback in the Post-lab Questionnaire

Question	Average Score + SEM (n=27)
How well did this lab help you to improve your knowledge on the endocrine system?	3.41±0.15
How well did this lab help you to improve your knowledge on the circadian rhythm?	3.41±0.14
How well did this lab help you to improve your knowledge on ELISA as a common medical laboratory method?	3.78±0.17
How well did this lab help you to improve your knowledge on data analysis with Microsoft Excel?	3.56±0.17

Figure 2. Distribution of students' responses to the post-lab questionnaire



about the endocrine system and circadian rhythm (3.41±0.15 and 3.41±0.14, n = 27). The last two questions regarding the ELISA method and data analysis received more positive student responses (3.78±0.17 and 3.56±0.17, n = 27). Students completed a lab report containing questions on topics covered in the lab. Lab reports were turned in the week following the lab experience and were graded by lab assistants. The average lab report grade was 96.2±0.7%.

Discussion

The student feedback was positive to the first two questions in the questionnaire about knowledge of the endocrine system and circadian rhythms. This meets our objective to enhance student

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understanding of the physiological functions that had been covered in lecture courses prior to the lab. The last two questions regarding the ELISA method and data analysis also received positive student responses, which meets our goal of strengthening the experimental and quantitative analytical skills of undergraduate students.

Student errors were common since none of the students had previously used the ELISA method. Many students were even unfamiliar with pipetting, which indicates that this lab module is a valuable addition to our physiology lab curriculum. However, due to the significant difference between evening and morning cortisol levels (visible even during color development, see Figure 1A), students were able to reach the correct statistical conclusion, which is that human cortisol levels are significantly different between morning and evening. We feel that this lab exercise is almost foolproof due to the naturally robust circadian rhythm fluctuations in cortisol levels. Therefore we believe that this lab is suitable for an undergraduate course with a laboratory component.

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