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M. H. Gillis, S. K. Duckett, J. R. Sackmann, C. E. Realini, D. H. Keisler and T. D. Pringle

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MEASUREMENT OF SERUM LEPTIN CONCENTRATIONS IN UNIVERSITY UNDERGRADUATES BY COMPETITIVE ELISA REVEALS CORRELATIONS WITH BODY MASS INDEX AND SEX

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Synthesized mainly in adipocytes, leptin is a peptide hormone that plays a key role in the regulation of body weight and composition. The serum leptin concentrations of 193 Singapore university medical and bioscience undergraduates aged 19–26 yr were measured using a competitive ELISA kit, and their leptin levels were correlated with sex and body mass index (BMI). Mean leptin levels were more than twice as high in females than in males of corresponding weight status, especially among females of healthy weight who exhibited levels that were 5.7 times higher. Overweight individuals generally demonstrated higher circulating leptin concentrations than healthy-weight and underweight participants. The differences in mean leptin levels between underweight and overweight males ($P = 0.006$), as well as between healthy-weight and overweight males ($P = 0.011$) were statistically significant. Comparison tests of leptin levels between healthy-weight and underweight females were highly significant ($P = 0.001$). Highly significant linear correlations between BMI and the logarithm of leptin concentration were observed in the female ($r = 0.44$) and male ($r = 0.36$) groups. These results reiterate the impact of gonadal steroids as mediators of the apparent sexual dimorphism in circulating leptin. The findings also corroborate evidence that adiposity determines leptin levels. This laboratory exercise has educational value for undergraduates by determining their BMIs, by alluding to the importance of maintaining healthy body composition, and by emphasizing the molecular mechanisms of body weight regulation and obesity, with special reference to leptin. This practical study also exemplifies the principles and applications of the competitive ELISA technique and integrates certain key concepts of physiology, molecular biology, immunology, and medicine.

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Key words: leptin; competitive enzyme-linked immunosorbent assay; body mass index; sex differences; correlation; undergraduates

Leptin is a 16-kDa protein expressed by the *ob* gene, is synthesized predominantly in adipocytes, and plays an important role in the regulation of body weight and composition, e.g., by reducing food intake and

increasing energy expenditure. This hormone signals to the central nervous system and peripheral organs about the body's nutritional status (e.g., fat storage level) and interacts with other metabolic hormones

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(20, 21, 28). Aberrations in the leptin-signaling pathway give rise to obesity in animals and humans (29). Specific polymorphisms in the leptin and leptin receptor genes have also been reported in some individuals with insulin resistance (6). Significantly elevated serum leptin levels are found in a considerable proportion of obese persons, implying resistance to endogenous leptin in human obesity (31). Serum leptin is a useful biomarker that reflects total body fat over a wide range of body mass indexes (BMIs) (36).

The objectives of this study were to measure leptin concentrations in serum samples from second-year medical and science (microbiology) undergraduates by use of a commercial kit based on competitive enzyme-linked immunosorbent assay (ELISA) and to correlate the serum leptin levels with BMIs and sex differences.

MATERIALS AND METHODS

Samples. From 1998 to 2000, 193 medical and bio-science undergraduates (111 males and 82 females), aged between 19 and 26 yr, volunteered to participate in the study. Each volunteer was required to personally register and sign in his or her name, age, and sex. Each subject's weight in kilograms and height in meters were measured, and the corresponding BMI or Quetelet index was calculated by dividing the weight by the square of the height. Venipuncture was performed by one of the authors (V. T. K. Chow), a registered medical practitioner. The students were given a detailed explanation of the collection process and potential hazards of blood specimens and the nature of the test on these specimens. About 5 ml of venous blood were collected from each student and serum was separated from clotted blood and stored at 4°C. Universal precautions were observed, including alcohol swabbing of skin, use of sterile needles and syringes, proper disposal of used needles in sharps bins, disinfection of biohazardous material (e.g., used swabs and syringes), and cleaning of table surfaces with alcohol or disinfectants. The experiments were performed with adequate supervision by experienced demonstrators, and students were reminded to handle the sera with utmost care and to presume that the specimens were biohazardous.

Leptin ELISA. The quantitative measurement of leptin in serum was performed using a leptin enzyme

immunoassay or ELISA kit (DRG Diagnostics, Marburg, Germany), according to the manufacturer's instructions. Briefly, 100 μ l of diluted leptin conjugate were dispensed into each well of the microtiter plate and incubated at room temperature for 1 h. The contents of the wells were shaken out and the wells rinsed three times with diluted wash solution. Into each appropriate well were dispensed 50 μ l of samples (diluted 1:5) and standards at concentrations of 0, 0.8, 1.6, 3.1, 6.2, 12.5, and 25 ng/ml. Fifty microliters of leptin antibody were then dispensed into the center of each well to achieve complete mixing, and the plate was incubated overnight at 4°C in a humidity chamber. The contents of the wells were shaken out, the wells rinsed thrice, and residual droplets removed. One hundred microliters of diluted second antibody were dispensed into each well and incubated at room temperature for 1.5 h. The contents of the wells were shaken out and the wells washed three times. One hundred microliters of horseradish peroxidase enzyme complex were dispensed into each well and incubated at room temperature for 45 min. Removal and washing of the wells were repeated before 100 μ l of tetramethylbenzidine substrate solution were added and then incubated at room temperature for 20 min. The enzymatic reaction was terminated by adding 50 μ l of sulfuric acid stop solution into the center of each well, and the absorbance at 450 nm was determined using an ELISA microtiter plate reader (Tecan, Salzburg, Austria). A standard curve was constructed by plotting a graph of the absorbance of each reference standard against its corresponding concentration in nanograms per milliliter. The leptin concentration of each serum sample was determined by using the corresponding absorbance to extrapolate the value from the standard curve and multiplying this by the dilution factor of 5. The manufacturer claims that the lowest detectable level of leptin distinguishable from the zero standard is 0.2 ng/ml and that the correlation of the enzyme immunoassay with a commercially available radioimmunoassay is 0.95. Interassay and intra-assay reproducibility was analyzed by the manufacturer by determining the coefficients of variation, which ranged between 3.6 and 7.8 and between 4.1 and 5.4%, respectively.

Statistical analyses. Statistical analyses were performed using SPSS software (version 10.0), including post hoc tests, multiple comparisons, Student's *t*-test,

one-way analysis of variance, regression analysis, Pearson correlation, and significance of correlation (two tailed).

RESULTS

The mean ages and their standard deviations were 21.0 ± 3.8 yr for males, 20.7 ± 3.3 yr for females, and 20.9 ± 3.6 yr for all subjects. The mean BMIs (in kg/m^2) and standard deviations were 22.3 ± 3.1 for males, 19.7 ± 2.0 for females, and 21.2 ± 3.0 for all subjects. In accordance with the international classification of weight status, the subjects were categorized as underweight, healthy weight, or overweight, based on BMIs of <20 , 20 to <25 , and ≥ 25 kg/m^2 , respectively. In the last category were two male subjects with BMIs of 32.2 and 36.3 kg/m^2 , who fulfilled the definition of obesity, i.e., ≥ 30 kg/m^2 (37).

Of a total of 193 sera tested, the lowest and highest serum leptin levels recorded were 0.2 and 71.5 ng/ml, respectively, implying broad limits of detection with the ELISA kit. To assess the reproducibility of the

assay, 28 selected sera were tested in duplicate and yielded closely matching absorbance readings. The high- and low-level controls and the reference standards were consistently valid in each batch of ELISA experiments.

Mean serum leptin concentrations were more than twice as high in females than in males of corresponding BMI categories, especially in the healthy-weight category where females exhibited levels that were 5.7 times higher (Table 1). In each sex group and in all subjects, overweight individuals generally demonstrated higher circulating leptin concentrations than healthy-weight and underweight participants. In addition, comparison tests of mean leptin levels between healthy-weight and underweight females were highly significant ($P = 0.001$). Statistical significance was also noted for the differences in mean leptin levels between overweight and underweight males ($P = 0.006$) as well as between overweight and healthy-weight males ($P = 0.011$).

Figure 1 depicts the scatter diagrams and (best fit) regression lines in the analysis of the relationship

TABLE 1
Distribution of BMIs and serum leptin concentrations by sex and weight status and statistical results of multiple comparisons and correlations

Sex/ Weight Status	<i>n</i>	BMI	[leptin]	<i>P</i> Values			Correlations	
				UW	HW	OW	Pearson (<i>r</i>)	2-Tailed sig. (<i>P</i>)
Male								
UW	25	19.02 ± 0.81	1.78 ± 2.66		0.686	0.006*	0.108	0.609
HW	66	22.06 ± 1.29	1.71 ± 1.09	0.686		0.011*	0.273*	0.026*
OW	20	27.30 ± 2.73	4.39 ± 5.82	0.006*	0.011*		0.181	0.446
All males	111	22.32 ± 3.07	2.21 ± 3.02				0.357†	0.000†
Female								
UW	50	18.49 ± 1.02	4.28 ± 5.97			0.001†	0.232	0.105
HW	30	21.39 ± 1.30	9.65 ± 14.16	0.001†		0.726	0.126	0.508
OW	2	25.15	9.83 ± 3.85	0.117	0.726		1.000†	
All females	82	19.71 ± 1.98	6.38 ± 10.02				0.439†	0.000†
Combined								
UW	75	18.67 ± 0.98	3.45 ± 5.23		0.998	0.506	0.043	0.712
HW	96	21.85 ± 1.32	4.19 ± 8.70	0.998		0.513	-0.003	0.980
OW	22	27.11 ± 2.67	4.88 ± 5.82	0.506	0.513		0.076	0.737
All subjects	193	21.21 ± 2.95	3.98 ± 7.20				0.076	0.296

Values of body mass indices (BMIs) and leptin concentrations are means ± SD. UW, underweight; HW, healthy weight; OW, overweight; [leptin], leptin concentration. *P* values are of multiple comparisons of log of [leptin] between weight categories; correlations are between log of [leptin] and BMI. Boldface figures highlight significance (sig.) values (*P*) and Pearson correlation coefficients (*r*) that are * statistically significant ($P < 0.05$) or † highly significant ($P \leq 0.001$).

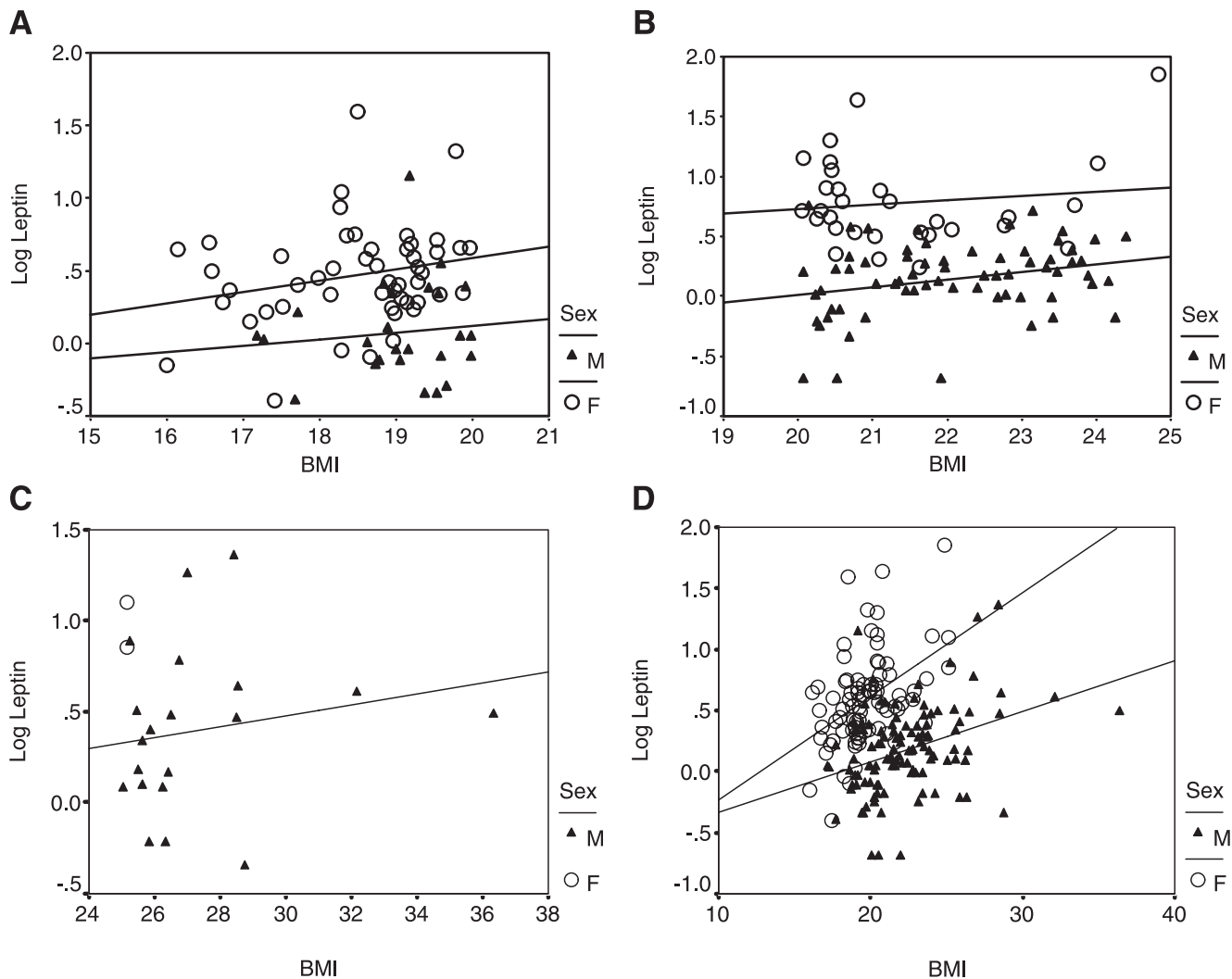


FIG. 1.

Correlation between body mass index (BMI) and logarithm of leptin concentration in male and female undergraduates belonging to the underweight (A), healthy-weight (B), and overweight (C) weight categories and to the whole cohort (D). Best fit regression lines for females are above those for males. However, no regression line could be estimated for 2 subjects in the overweight female category (C).

between BMI and the logarithm of leptin concentration for both sexes, and Table 1 displays the relevant Pearson correlation coefficients and their degrees of significance. A moderately close positive linear correlation ($r = 0.44$) that was highly significant ($P < 0.001$) was observed in the female group, whereas an albeit weaker correlation ($r = 0.36$) that was also highly significant ($P < 0.001$) was noted in the male group, especially among males of healthy weight ($r = 0.27$, $P < 0.05$).

DISCUSSION

Overall, the anthropometric parameters and BMI percentiles of this young adult cohort in Singapore were generally lower than those of equivalent European and American cohorts (9). Interestingly, this postpubertal and young adult cohort of university undergraduates exhibited sex profiles of leptin concentrations that mirror those of documented cohorts of recruited older adult subjects. The occurrence of the marked

sex dichotomy in leptin levels also concurs with previous studies and may be explained by differences in sex hormones (14, 26).

Besides its role in regulating energy homeostasis, leptin also modulates the hypothalamo-pituitary-gonadal axis and signals the brain about the body's preparation for pubertal development, sexual maturation, and reproduction (15, 22, 32). In healthy women, the ultradian fluctuations in circulating concentrations of leptin show pattern synchrony with those of luteinizing hormone and estradiol (25). Moreover, maternal leptin levels are elevated during late pregnancy and at birth (32). Cento et al. (4) found significantly lower leptin levels in postmenopausal obese women compared with their premenopausal counterparts. Circulating leptin correlates inversely with testosterone levels in older African American men and in pubertal males (18, 30). At later stages of pubertal development, circulating leptin concentrations rise significantly in female children but decline in male children. All of these observations underscore the impact of gonadal steroids as mediators of this apparent sexual dimorphism in circulating leptin levels (18, 33).

In agreement with other studies involving cohorts of different age groups and ethnic backgrounds, our results corroborate evidence that adiposity determines leptin concentrations (5, 10, 35). The significantly higher leptin concentrations in overweight or obese compared with lean or normal-weight people suggest that the effect of leptin on appetite control is diminished or absent. This is compatible with animal studies in which the administration of leptin decreases food intake, adiposity, and body weight in leptin-deficient *ob/ob* mice (29) but not in leptin-insensitive *db/db* mice (22).

The serum samples were not collected from fasting subjects, because it has been shown that serum leptin levels remain unchanged during fasting (3). Notwithstanding this, circulating leptin levels may be influenced by other factors, e.g., body composition (5), overeating (24), exercise (34), regular physical training (11), ethnicity (27), and mutations in other genes (12, 17). Although touted as the antiobesity hormone, clinical trials employing leptin reveal varying efficacy, i.e., weight loss in some, but not all, obese candidates (13, 23). Discoveries in recent years have unveiled

other novel peptide regulators of food intake that participate in new pathways, some of which affect leptin, e.g., agouti-related peptide, cholecystokinin, corticotropin-releasing hormone, galanin, mahogany protein, melanin-concentrating hormone, melanocortins, neuropeptide Y, and orexins (1, 2, 7, 16, 17).

Under the supervision of laboratory technologists, some student representatives were involved in the actual experimentation, data interpretation, and graph construction. Feedback information indicated that this hands-on experimental exercise was beneficial and useful to the students in the following ways:

- to learn the formula for calculating their BMI to determine their weight status and the definitions of underweight, healthy weight, overweight, and obesity
- to demonstrate the collection of blood and the use of serum as a specimen for testing the concentration of an endogenous antigen
- to understand the scientific and technical basis of competitive ELISA, a modification of the popular and quantitative enzyme immunoassay
- to practice and perform statistical tests on variables, i.e., leptin concentration, BMI, and sex
- to know their own serum leptin levels and correlation with BMI and sex
- to specifically highlight the role of the important regulatory peptide leptin
- to learn and understand more about the complex physiology of appetite, nutrition, metabolism, and weight regulation
- to increase awareness of the burgeoning epidemic of obesity worldwide and the urgent need to elucidate the intricate molecular pathophysiology underlying obesity
- to illustrate how an experiment is designed to ascertain the relationship between different variables, even though the students did not design this study.

The student learning process may be assessed on the basis of practical aspects and knowledge acquisition. Practical aspects include:

- how to determine body weight and height
- how to perform enzyme immunoassay (e.g., addition of serum specimen, antibody, enzyme, and substrate; washing steps; use of spectrophotometer to measure absorbance; and importance of controls)
- how to determine leptin concentration by plotting a standard curve from reference standards
- how to collate data and employ statistical tests to analyze variables to determine their significance and relationships.

Knowledge acquisition includes:

- formula for calculation of BMI and weight status definitions
- principles of competitive ELISA
- different statistical tests and their appropriate applications
- importance and role of leptin in regulation of body weight
- complexity of weight regulation, which involves not just leptin but a host of other molecules and factors that interact with one another.

Evaluation of student performance may be achieved using one or more formats, such as reports in laboratory exercise books, postlaboratory tutorials, continuous assessments, and formal tests or quizzes based on theoretical or experimental components (e.g., actual ELISA microtiter test plate).

Although this laboratory exercise was not part of a regular curriculum within medical and life science undergraduate education, this study may form the basis for developing new curriculum. Notwithstanding that it has already been documented in other cohorts that leptin levels are influenced by both BMI

and sex, this project was initiated to explore its suitability as teaching methodology for illustrating the physiology of leptin. Our experience has confirmed the feasibility of adopting this experiment as a regular part of the curriculum. However, the experiment may be further modified and improved by analyzing the effects of factors on leptin other than BMI and sex (e.g., circadian rhythms, food intake, exercise, physical fitness) or by using other methods of body fat assessment (e.g., body circumferences, skinfold thicknesses).

In conclusion, this laboratory exercise has personal interest value for undergraduates by their determining their own BMIs, which indicate whether they have healthy weight or are underweight or overweight. It also acts as an instructive health education exercise that alludes to the importance of maintaining healthy body composition. This practical study emphasizes recent advances in understanding the molecular mechanisms underpinning the regulation of body weight and the development of obesity with particular reference to the role of leptin. Furthermore, it reinforces the biochemical principles of the competitive ELISA technique, a modification of ELISA that is a widely applied, robust, sensitive, and automated tool in medical diagnosis. Comparisons can also be made between ELISA and other methods, such as radioimmunoassays (8). Coupled with appropriate data interpretation, a critical educational goal is to integrate the learning of certain key scientific concepts of biochemistry, molecular biology, immunology, and medicine, especially in relation to body weight regulation and obesity, which is an increasingly prevalent public health problem even in developing countries (19).

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