CHAPTER 8

WOMEN AS PARTICIPANTS IN CONTROLLED DIET STUDIES

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Historically most medical research has been conducted using male subjects. Women have often been excluded from clinical studies for many reasons, including protection from risk to a potential fetus or nursing infant, lower rates of the disease under study, and possible confounding from menstrual cycle variations. Also, cost constraints have discouraged gender-specific data analyses, which require larger sample sizes to obtain sufficient statistical power in each subgroup.

Results of research on men, however, cannot always be extrapolated directly to women. For example, important differences in drug metabolism or the natural history of disease can occur in women because of hormonal changes associated with the menstrual cycle or menopause. These natural biological events can affect many physiological systems and can complicate study design and interpretation, but they are important characteristics of the recipients of the resulting health care. Recent guidelines of the National Institutes of Health mandate inclusion of women in health research unless there are compelling arguments to the contrary (1). If there is evidence of significant gender differences in response, the research design and sample size must be able to answer the research question separately for each gender. In this way, research findings will apply to both men and women, and all persons can benefit.

This chapter addresses female hormone status and other related issues in the specific context of well-controlled feeding studies.

The ovarian cycle, methods of identifying the phases, oral contraceptives, menopause, and hormone replacement therapy are reviewed. Dietary issues and effects of the menstrual cycle on physiological systems that influence the results of nutrition research are discussed. Approaches are also presented for improving research design, data collection and analysis, recruitment strategies, and subject management. (Readers wishing to obtain a more comprehensive, general overview can consult DA Krummel and PM Kris-Etherton, eds, *Nutrition in Women's Health,* Gaithersburg, Md: Aspen Publishers, Inc; 1996.)

HORMONAL STATUS: MENARCHE TO MENOPAUSE

Normal Menstrual Cycle

The onset of menstrual cycles, *menarche,* typically occurs at 12 years to 13 years of age; the normal range extends from 8 years to 18 years (Table 8–1). Menarche is one step in the process of puberty, which can span several years (2–4). Although young girls and adolescents are seldom enrolled in controlled diet studies, investigators should consider whether menarche represents a possible confounding factor in study design.

The normal menstrual cycle is a complex interaction of hypothalamic, pituitary, and ovarian endocrinology (5–8).

TABLE 8-1

Reproductive States in Women

State	Typical Age Range[1] (yr)	Menstrual Cycles	Other Characteristics
Prepuberty	0–12	None	Physically immature (Tanner Stage 1)
Puberty	8–18	Irregular; anovulatory at first	Physical maturation (Tanner Stages 2–4) Peak height velocity precedes menarche Menarche: menses begin
Childbearing potential	12–50	Regular	Physically mature (Tanner Stage 5) Pregnancy; Lactation Infertility; Amenorrhea (primary, secondary) Contraceptive use
Menopause: Perimenopausal	45–55	Irregular; still ovulatory	Endogenous hormone levels reach new ''set'' points Multiple physical and other changes (''climacteric'') Hormone replacement therapy
Menopause: Postmenopausal	45+ (average 52)	None	Natural menopause Surgical menopause (with/without oophorectomy) Hormone replacement therapy

[1]Age ranges overlap to indicate the range of normal biological variation.
Sources: Gerhard I, Heinrich U. Puberty and its disorders. In: Runnebaum B, Rabe T, eds, *Gynecological Endocrinology and Reproductive Medicine,* Vol 1, *Gynecological Endocrinology,* New York, NY: Springer-Verlag; 1994. Jones RE, *Human Reproductive Biology,* 2nd ed, New York, NY: Academic Press; 1997.

Under hypothalamic control, the anterior pituitary produces two main gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and other hormones that affect the cycle to a lesser degree. The ovaries produce the sex steroids, estrogen (estradiol) and progesterone.

The normal cycle averages 28 days in length, with ovulation occurring between the 13th day and l5th day when the cycle is numbered from the first day of menses (Figure 8–1a). The cycle can vary within and among women from 25 to 32 days in length. Cycles of less than 21 days or greater than 35 days may not represent normal ovulation.

The cycle can be divided broadly into two major phases: follicular and luteal. The follicular phase begins with the onset of menstruation, which is really the culmination of the preceding cycle. During the early follicular phase, all serum hormone levels are low (Figure 8–1b). Then estrogen begins to rise, stimulating FSH production and the maturation of an ovum (egg follicle). Rising estrogen levels stimulate LH production, and both LH and FSH rise abruptly. On release of these pituitary hormones, estrogen levels drop sharply. The peak of LH and FSH marks the end of the follicular phase and the beginning of the luteal phase. Within 1 day of the peak, ovulation occurs; the ovum is released from the ovary. Estrogen begins to rise again and progesterone increases to maximum about 5 to 7 days after ovulation. Estrogen and progesterone levels decline toward baseline during the late luteal phase and menstruation begins as the lining of the endometrium is sloughed.

Documenting the occurrence of ovulation can be important in studies of specific cycle phases. Some authors divide the cycle into four phases: menstrual, follicular, ovulatory, and luteal. Cycles within an individual woman can be classified as ovulatory or anovulatory or as having a short luteal phase (ie, lasting fewer than 10 days) (9).

Chronological age can also affect menstrual cycle parameters in actively cycling women. Older premenopausal women (37–45 yr) have been found to have higher serum gonadotrophin (LH and FSH) levels, lower follicular phase length, later ovulation, and increased endometrial thickness compared with younger women (21–25 yr) (10).

Cessation of menses is almost always the first sign of pregnancy, although a small proportion of women continue to bleed periodically. After delivery there is considerable variation in the time before regular ovulatory cycles resume. This interval generally is longer in lactating women.

Methods of Phase Identification

Many of the techniques for monitoring reproductive function and detecting ovulation were originally developed for treating infertility, but they also have facilitated the study of

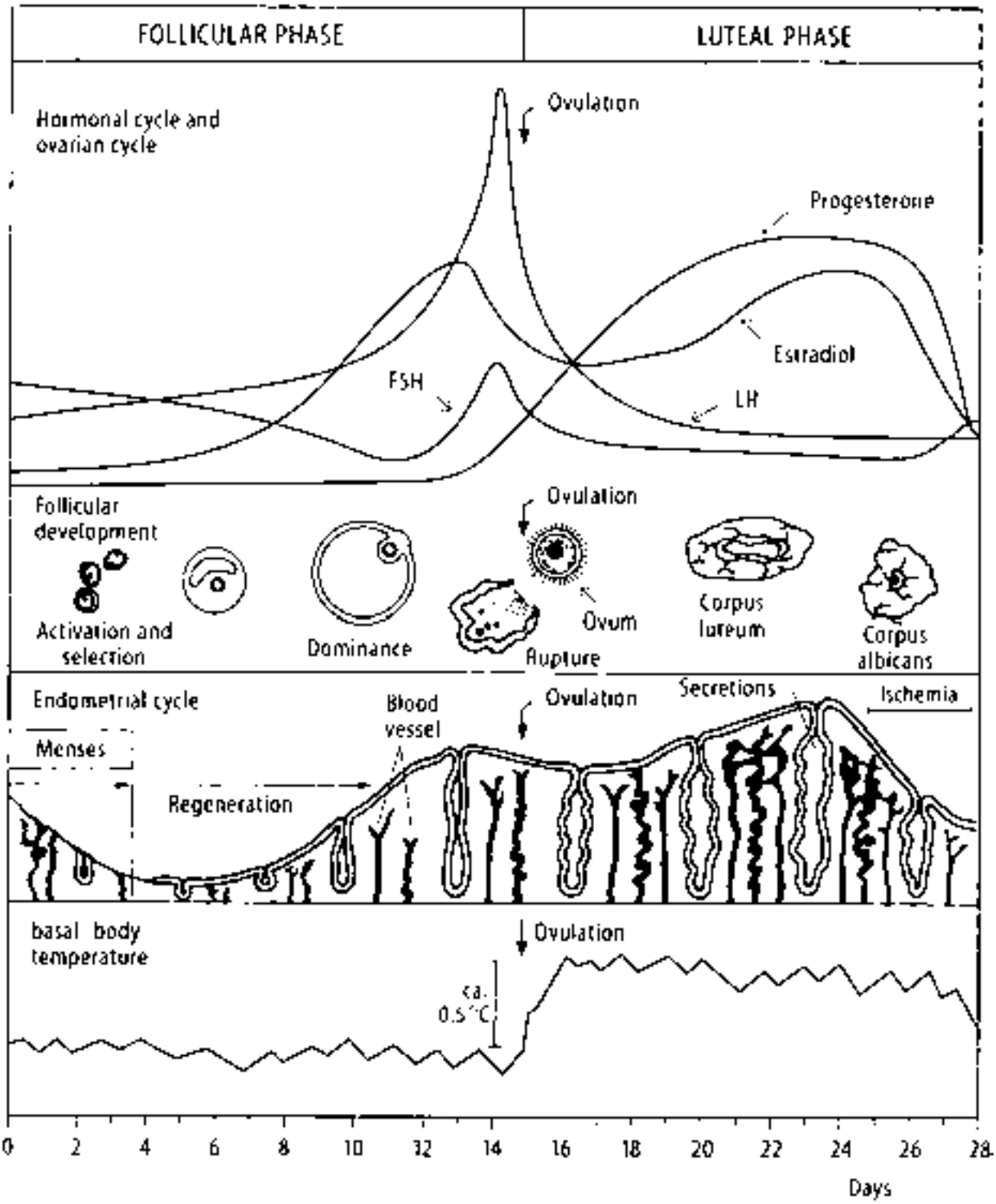

FIGURE 8–1a. Circulating hormone levels, follicular development, endometrial changes, and basal body temperature during the menstrual cycle. Reprinted with permission from: Weinbauer GF, Nieschlag E, Hormonal regulation of reproductive organs. In: Greger R, Windhorst U, eds, *Comprehensive Human Physiology,* Vol 2, New York, NY: Springer-Verlag; 1996:2243.

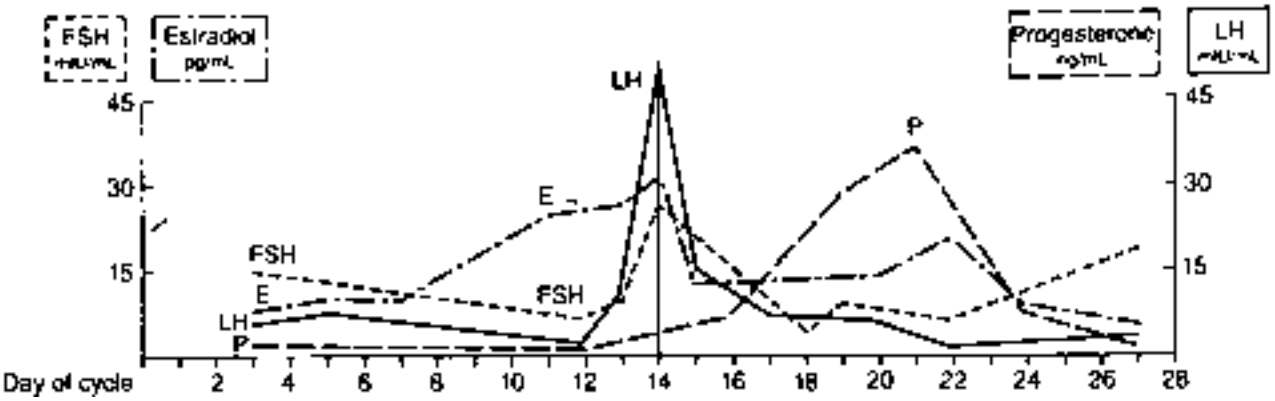

FIGURE 8-1b. Concentrations of circulating hormones during the menstrual cycle. Reprinted with permission from Grunwald K, Rabe T, Keisel L, Runnebaum B, Physiology of the menstrual cycle. In: Runnebaum B, Rabe T, eds, *Gynecological Endocrinology and Reproductive Medicine,* Vol 1, *Gynecological Endocrinology*, New York, NY: Springer-Verlag; 1994:132.

normal hormone status and menstrual cycle phases. Identifying specific phases of the menstrual cycle enables investigators to evaluate outcome variables as they relate to hormone fluctuation and to synchronize sample collection with the corresponding phase. Currently available methods for monitoring the phases and stages of the cycle include direct assay of hormones or their metabolites in serum, urine, or saliva; ultrasound; and observation of the physiological changes that occur in response to hormonal changes (menses, basal body temperature, and cervical mucus consistency) (11, 12).

Serum

Many of the assays for serum hormone levels are accurate and reliable, and they are readily conducted in most clinical laboratories. Researchers considering their use should evaluate the accuracy, precision, and reproducibility of commercial kits or other analytical methods when planning the study protocol. Laboratory quality control procedures appropriate for clinical practice and patient treatment may not be sufficiently stringent for research purposes. The monitoring techniques discussed here can be employed to identify the days most crucial for analysis of serum levels if pinpointing stages of the cycle is of interest. However, daily blood sampling is seldom done because of the high cost of analysis and the burden on participants.

The phases of the ovulatory cycle generally are documented using serum progesterone levels, which are usually less than 1 ng/mL in the follicular phase and rise to above 10 ng/mL in the luteal phase. Values above 5 ng/mL generally indicate that the subject is in the luteal phase of an ovulatory cycle (13).

Urine

Daily urine sampling is feasible for research studies, and new technologies make kit assays practical (14, 15). Regularly timed daily urine specimens can be assayed for metabolites of steroid hormones. Urinary hormone levels have less diurnal variation than serum levels and do not reflect the pulsatile secretion rate of some hormones. This can be an advantage over serum sampling if average levels are of interest, but peaks may be missed. The sample usually is the first void of the day; results are normalized to the urinary creatinine concentration.

During the luteal phase, urinary levels of pregnanediol, a metabolite of progesterone, reach 4 to 6 ng per 24-hour collection. Values above 2 ng per 24 hours indicate the luteal phase of an ovulatory cycle (10).

In-home ovulation prediction kits contain a monoclonal antibody specific for LH. An enzyme-linked immunosorbant assay reaction (ELISA) elicits a color change proportional to the level of LH in the urine. If baseline observations are established before ovulation is expected, the LH surge will be apparent. Testing for 10 consecutive days is sufficient to detect 95% of LH surges. Strict adherence to the manufacturer's instructions is necessary for accurate results. Samples can be stored in the refrigerator for later analysis, if necessary, but the analysis must be done at room temperature. Samples should be collected at the same time each day, with beverages restricted 1 or 2 hours immediately preceding collection so the urine is not too dilute. The various commercially available kits differ in details such as: cost, the number of days the kit can be used, the length of time for the color change to stabilize, the time of day the urine sample is collected, and the length of time between a positive result and the occurrence of ovulation. Examples of such kits include: First Response Ovulation Predictor Test, Carter-Wallace, Inc; Answer Ovulation Test Kit, Carter-Wallace, Inc; and pharmaceutical house generic brands. (Examples are provided for information purposes only and do not represent endorsements by either The American Dietetic Association or the National Heart, Lung, and Blood Institute.)

Saliva

Unconjugated steroid hormones diffuse freely and appear in saliva in small amounts proportional to serum levels (16, 17). Salivary steroids are 0.5% to 2% that of serum (18). Concentrations are not affected by the rate of saliva produc-

tion, but any bleeding in the mouth will greatly increase steroid levels in the saliva.

Daily sampling of saliva is feasible in the context of research protocols. Two to 5 ml of fasting saliva is needed for the assay, which is relatively simple and inexpensive (19, 20). Specimens should be collected as participants arise, before they eat, or brush or floss their teeth. Citric acid applied to the tongue will increase saliva flow without affecting the assays (12). Samples can be stored in the home freezer for 1 month and are stable for 6 months at −20°C. Testing the saliva samples for hemoglobin or another marker of bleeding gums would allow contaminated samples to be discarded.

Ultrasound

Ultrasound can be used to observe the physiological changes in the ovaries and uterus during the menstrual cycle (21). During the follicular phase the size of the dominant follicle and the thickness of the endometrium correlate with the serum levels of estradiol (see Figure 8–1a). At ovulation the dominant follicle will typically have a maximum diameter of 20 mm to 23 mm, ranging from 14 mm to 29 mm. This coincides with the LH peak. Ultrasound can document ovulation and differentiate the cycle phases; endometrial thickness reaches a peak during the luteal phase. High cost and potential discomfort to the subject must be taken into account when investigators design protocols requiring ultrasound evaluations.

Menstrual Calendars

The appearance of the menses is the most commonly used parameter for determining the phases of the menstrual cycle. To control for hormone cycling, many research studies use the phase immediately following menses (ie, early follicular phase), when the hormone levels are low and steady. This period lasts about 6 days before estrogen begins to increase, but there is much variation within and among women. Phases can be identified retrospectively by counting back 14 days from the menses to determine the approximate day of ovulation. There is less variation within and among women in length of the luteal phase. For women with regular cycles, these methods can effectively approximate the hormone cycle. However, ovulation is a more critical event than menstruation in terms of hormonal surges and changes that affect metabolic parameters.

The menstrual calendar is a convenient and inexpensive means of collecting necessary data. It is easily incorporated into many types of study protocols. (An example is provided in Exhibit 8–1.)

Basal Body Temperature

Measurement of basal body temperature (BBT) can be used to identify ovulation and demarcate follicular and luteal phases (see Figure 8–1a and Figure 8–2). A rise in BBT occurs from 2 days before to 3 days after the LH peak and is a reliable indicator that ovulation has occurred (22, 23). Least-squares methods of analyzing BBT data yield good correlations with LH concentration and can generate detailed data on menstrual cycle type (normal ovulatory, short luteal phase, or anovulatory) and luteal phase length (24).

BBT is a simple method of monitoring the menstrual cycle. It is well accepted by subjects, is noninvasive, and is inexpensive. However, the method relies on the subjects to monitor and record temperatures accurately. Mercury thermometers are fragile, take 3 to 5 minutes to register an accurate temperature, and are easily misread. The temperature must be taken on awakening, before participants arise. At this time of day, body temperatures are approximately 1.0°C (1.5°F) lower than usual. Oral, vaginal, or rectal temperatures can be used, but the same site must be used consistently. Irregular sleep and emotional stress can invalidate the measurements. (An example of a BBT chart is provided in Figure 8–2.)

Computerized electronic devices combine the BBT and the calendar methods of identifying the fertile and infertile periods of the menstrual cycle (21). This eliminates the need for subjects to read and record temperatures. The general operation of these devices follows a similar pattern. The Bioself 110 (Bioself Distribution SA, 7 Avenue de Thorex, 1226, Geneva, Switzerland) is an example of such a device. (This example is provided for information purposes only and does not represent an endorsement by either The American Dietetic Association or the National Heart, Lung, and Blood Institute.) The user inputs the start of each menses by pushing a button. During the first month of use the program assumes a 28-day cycle. In subsequent months the program becomes increasingly more accurate for the individual user. The microprocessor subtracts 18 days from the length of the shortest cycle recorded and, with a steady red light, indicates this day as the beginning of the fertile period (late follicular phase). A display such as a flashing red light indicates the expected ovulatory phase. After the temperature rise following ovulation, another display (such as a green light) indicates the luteal phase. If ovulation does not occur during this cycle, and the temperature does not rise, the ovulatory phase display persists until the onset of menses is indicated by the user. The memory stores the temperatures and the displays each day for 2–3 months and the cycle lengths for 6 months. The devices can be programmed to accept temperature measurement at restricted times of the day, thus promoting a consistent daily time that the BBT is measured. Twenty-four hours after the last reading, the device emits a reminder tone signal. Data usually can be retrieved in graphic form, permitting review of the temperature charts and cycle lengths. Identification of the ovulatory period using electronic calendars correlates strongly with the ultrasound documentation of maximum follicular diameter (25) and the serum LH peak (26). The technology for such devices is improving rapidly and can be expected to yield modifications that would be useful in the research setting.

Cervical Mucus

Klaus (27) described monitoring of cervical mucus, used extensively in fertility and contraception techniques. The

EXHIBIT 8-1

Example of a Menstrual Calendar[1]
DELTA
Menstrual Calendar

Subject number:_____ Usual cycle length:_____days

On the calendar below put an X in the block corresponding to the first day of your period (first day of bleeding). Check (√) each day that bleeding continues enough to require a pad or tampon. Turn in this calendar to study personnel at your last blood drawing.

SEPTEMBER						
SUN	**MON**	**TUES**	**WED**	**THU**	**FRI**	**SAT**
26	27	28	29	30		
OCTOBER						
SUN	**MON**	**TUES**	**WED**	**THU**	**FRI**	**SAT**
					1	2
3	4	5	6	7	8	9
10	11	12	13	14	15	16
17	18	19	20	21	22	23
24	25	26	27	28	29	30
31						
NOVEMBER						
SUN	**MON**	**TUES**	**WED**	**THU**	**FRI**	**SAT**
	1	2	3	4	5	6
7	8	9	10	11	12	13
14	15	16	17	18	19	20

Note any *abnormalities* in your periods during the 8 weeks of the diet study with a √.

		Period[2]		
		1	**2**	**3**
Start date	Earlier than usual	_____	_____	_____
	Later than usual	_____	_____	_____
Cramps	Worse than usual	_____	_____	_____
	Not as bad as usual	_____	_____	_____
Premenstrual Syndrome (PMS)[3]	More severe than usual	_____	_____	_____
	Less severe than usual	_____	_____	_____

[1]Adapted from *Manual of Operations for the DELTA Program* (74).
[2]Most women will have only 2 periods during the diet study. However, 3 columns are provided because occasionally someone may have 3 periods within this time.
[3]PMS is a constellation of symptoms that usually occurs 7–10 days before menstruation and that disappears with the start of the new cycle. Physical symptoms include bloating, breast swelling, pelvic pain, headache, ankle swelling, and bowel changes. Psychological symptoms include irritability, aggressiveness, anxiety, tension, and changes of libido.

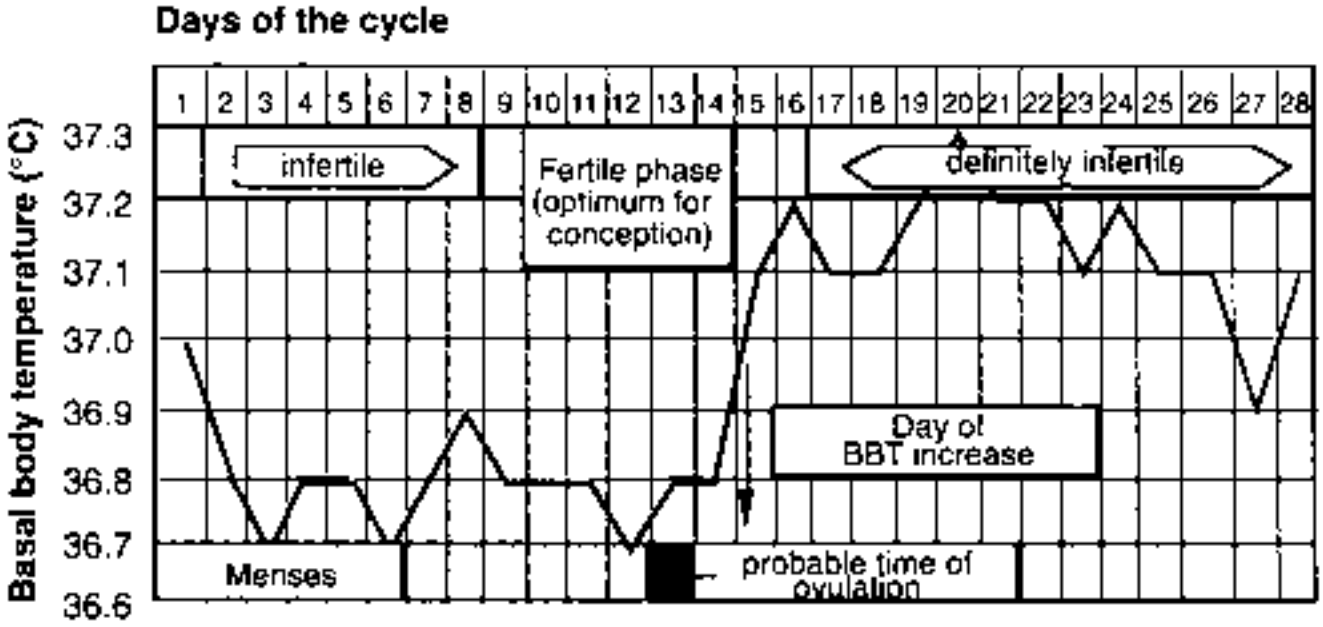

FIGURE 8-2. BBT chart for determining the time of ovulation. Reprinted with permission from Rabe T, Runnebaum B, Contraception. In: Runnebaum B, Rabe T, eds, *Gynecological Endocrinology and Reproductive Medicine,* Vol 1, *Gynecological Endocrinology*, New York, NY: Springer-Verlag; 1994:405.

mucus is a hydrogel of two components: one of high and one of low viscosity. The proportions of these components vary directly with hormone changes. ''Type E'' (estrogenic) mucus is thin, slippery, and acellular; it is in high proportion before and at ovulation, correlating with the LH surge in response to estrogen. ''Type G'' (gestagenic) mucus is thick, sticky, and dense; it occurs after ovulation in response to progesterone.

Subjects can be taught to identify the onset of mucus production and the changes in consistency. These techniques could be applied to research studies to identify ovulatory cycles and the phases accurately.

Steroidal Contraceptive Medications

Ninety percent of women at risk of pregnancy use some form of contraception. About 28% use steroidal contraceptives (28). These act to inhibit ovulation by negative feedback on LH and FSH so that the ovum does not develop. Steroidal contraceptives also render the cervical mucus and the endometrium less supportive of fertilization and pregnancy.

Three major classifications of oral contraceptives (OC) contain both estrogen and progestin: (1) *monophasic,* with a constant amount of the two hormones in each tablet; (2) *biphasic,* with a constant amount of estrogen but with progestin lower for the first 10 days and higher in the last 11 days; (3) *triphasic,* with constant or varied levels of estrogen and varied progestin. The purpose of varying the hormone levels is to mimic the natural cycle. Recently, oral contraceptives that contain only progestin have become available.

Not all contraceptive drugs are taken by mouth. Long-acting steroidal contraceptive systems that deliver progestogens continuously over an extended period of time are also in use. They include injectable depot formation; subcutaneous polymer implant; vaginal rings; medicated intrauterine devices; and injectable polymeric formulations (29).

Steroidal contraceptive use, which usually represents OC use, must be examined in light of protocol criteria to determine whether it is a potential confounding factor or effect modifier. Use is not a problem for some studies but is highly problematic for others. A problem situation develops if the steroidal contraceptive has direct biological effects on the study outcome variables, if the contraceptive modulates the influence of diet on the outcome variables, or both. If it is necessary or desirable to enroll steroidal contraceptive users in the study, confounding can be minimized through crossover designs or random balanced assignment of users and nonusers to treatment groups.

Menopause and Hormone Replacement Therapy

Menopause is the permanent cessation of menstrual cycles (Table 8–1). It occurs typically between ages 45 and 55 years, with small percentages of women experiencing menopause before 45 or after 55 years of age (3). As natural menopause approaches, cycling becomes irregular, then ceases—a process that can take months or years. During this time a decline in estrogen and an increase in FSH occur. For several years before cycles decrease, ovarian estradiol and progesterone production diminishes. This decreases the negative feedback inhibition on the hypothalamic pituitary system, and FSH levels gradually rise. The ovarian follicles

become increasingly refractory to elevated concentrations of FSH.

Postmenopausal ovarian estrogen production is minimal. The adrenal androgens, especially androstenedione, can be converted in adipose tissue to estrogens; the degree of adiposity correlates with the amount of conversion. The ovaries continue to produce testosterone and androstenedione at or near premenopausal levels. The change in estrogen:testosterone hormone ratios may lead to increased facial hair and other masculinized characteristics.

Relatively large numbers of women undergo surgical menopause following hysterectomy procedures. Depending on medical considerations the ovaries may either be removed (oophorectomy) or left intact. Ovarian function then may be sustained as before but may in some cases decline more rapidly than normally would be expected.

The acute drop in serum estrogen levels from natural menopause or surgical oophorectomy can cause instability of the hypothalamic thermoregulatory set-point, leading to vasomotor flushes or ''hot flashes.'' During a hot flash cutaneous blood flow and skin temperature increase; core body temperature falls. Blood pressure remains stable. Plasma LH rises to peak at about 12 minutes after onset of the flush. These flushes can interfere with sleep and contribute to irritability, reduced concentration, and impaired memory. It is not known how these events—either through physiologic mechanisms or by affecting compliance—might confound a study that includes perimenopausal women.

For some (but not all) protocols, confounding from these issues and from shifting hormonal levels will render the perimenopausal age range unsuitable for recruitment. In this case investigators are advised to recruit participants who are clearly pre- or postmenopausal as determined by blood FSH levels or by questionnaire (ie, about the number of months or years since last menstrual period).

An increasing number of women who reach menopause choose to take hormone replacement therapy, either to treat direct symptoms of menopause or to retain the health benefits of estrogen. Many different hormone preparations are available. If the formulation contains both estrogen and progestin, menstrual cycles will continue in a woman who retains an intact uterus, but ovulation does not occur.

Chronological age, menopausal age, hormonal status, and hormone replacement are factors that may affect disease risk factors and response to diet. All of these need to be carefully considered in the design and implementation of research studies. Potential study participants can be categorized according to whether the menopause was natural or surgical (oophorectomy); whether hormones are replaced; and whether the hormones are provided as estrogen alone or with progestin (30), for how long, and by what routes of administration (31). How menopause and replacement hormones affect risk of chronic disease (such as cancer, heart disease, and osteoporosis) has become a high research priority now that greater numbers of women are living 30 years or more after the menopause (32, 33).

Effects of the Menstrual Cycle on Physiologic Systems

Many physiological systems are affected by the hormonal cycle and menopause. This section presents some interesting examples, many of which are pertinent to well-controlled feeding studies.

Lipids

Serum cholesterol levels appear to be affected by the menstrual cycle. Total cholesterol is approximately 10% higher in the follicular phase, especially close to ovulation, than in the menstrual and luteal phases (34–36). Cholesterol is a substrate for steroid synthesis, and it is biologically plausible that the hormone cycle would affect serum cholesterol levels. The evidence for menstrual cycle effects on serum triglycerides is inconclusive (37, 38).

Serum low-density lipoprotein cholesterol and triglycerides rise with declining sex hormones after menopause, and high density lipoproteins fall, independently of age and other factors (39). Unopposed estrogen replacement is the optimal treatment for maintaining serum lipids at premenopausal levels, but combined estrogen/progestin replacement also is highly effective (39).

Blood Pressure

Systolic blood pressure is higher in the luteal phase, with a gradual increase until the day of menstruation. Thereafter, blood pressure drops sharply and remains low through the follicular phase. Variability among women is wide (40, 41). Conflicting results between studies may be caused by hormonal changes that do not directly affect blood pressure but instead affect response to stress. For example, mental stress tests applied to normally menstruating women caused greater increases in heart rate and systolic and diastolic blood pressure during the luteal phase than in the follicular phase (42). Without applied mental stress, no differences were observed between the phases. This suggests it is reaction to stress, not blood pressure per se, that varies with the menstrual cycle.

Regensteiner et al (43) found that hormone replacement therapy (HRT) as combined estrogen and progestin maintained blood pressure at premenopausal levels, whereas lack of HRT or estrogen alone could not prevent the rise in blood pressure frequently seen in postmenopausal women. The relationship of dietary calcium and blood pressure also may be different in pre- vs postmenopausal women (44).

Sodium Balance

Urinary sodium balance may fluctuate during the luteal phase because the progesterone surge at ovulation can inter-

fere with the action of aldosterone on the renal tubules, causing net sodium loss. Aldosterone levels may increase in response to the interference, whereas the progesterone levels are declining. This leads to sodium retention and low urine sodium levels (45).

Caffeine Clearance

Caffeine clearance may differ between the phases of the cycle. Lane et al found an 11% reduction in caffeine clearance during the luteal phase (46). As clearance had a negative correlation with estrogen levels, it may also be reduced in persons taking estrogen replacement or oral contraceptives. This difference is not enough to cause caffeine intoxication but may affect the results of metabolic studies.

Fluid Balance

Some women complain of ''bloating'' (a feeling of swelling) premenstrually, but weight gain does not necessarily accompany this complaint, suggesting a shift of fluids between compartments. Oian et al, studying transcapillary fluid dynamics, found a reduction of the plasma and interstitial colloid osmotic pressures in the luteal phase, which may be from simple dilution and reduction in total body protein mass (47). Changes in the metabolism of plasma proteins during the menstrual cycle may account for this observation, because there was no external loss of plasma protein. Mechanisms are as yet unknown.

Vokes et al (48) demonstrated that osmotic thresholds for thirst and vasopressin secretion decreased during the luteal phase. Plasma sodium also dropped 2 mEq/L during the luteal phase. These changes were small but statistically significant. Therefore, a true shift may occur in the setting of the osmoregulatory system between follicular and luteal phases.

Energy Expenditure, Energy Intake, and Eating Patterns

Several investigators (49–52) have reported increased energy expenditure during the luteal phase compared to the follicular phase. In particular, Webb reported a mean increase of 9% in daily energy expenditure in the luteal phase (49). Intraindividual variation was also greatest in the luteal phase. Bisdee observed a consistent pattern of 7% maximum increase in 24-hour energy expenditure in the late luteal phase and lowest expenditure in the late follicular phase (ie, immediately before preovulation) (50).

Spontaneous food intake also may vary across the menstrual cycle and between monthly cycles. Protocols requiring information on typical dietary patterns may thus require multiple dietary assessments during each phase for an accurate picture of overall intake. Several outpatient studies of food intake across the menstrual cycle have reported energy intake during the luteal phase that was higher by 100 or 200 calories per day (53, 54). In contrast, a study that confined subjects to a metabolic unit and controlled for activity found no significant changes in energy intake over the menstrual cycle (55). Food intake was monitored inconspicuously, but the inpatient setting may have diminished spontaneous intake. An increased intake of sucrose—in the form of candy, chocolate, and soft drinks—was observed in the luteal phase. Fluid intake, as food water and beverages, increased during the luteal phase, and urine volume was correspondingly higher; dry weight of foods was equal.

Gastrointestinal Function

Study results of the menstrual cycle's effects on gastrointestinal function are conflicting (56–59). Some report slower mean transit time in the luteal phase, whereas others find no difference with phases of the cycle. Subjects eating a controlled diet had increased starch absorption, decreased breath hydrogen, and decreased stool weight (dry and wet) in the luteal phase. No effects have been found of the cycle phases on gastrin, somatostatin, oxytocin, and gastric inhibitory polypeptide (60). Cholecystokinin, however, is higher in the luteal phase (61). Its expected effects, such as increased gallbladder contractility, increased gut motility, and increased satiety, are counteracted by progesterone, which also has high levels during the luteal phase. This competition may contribute to the inconsistent results reported.

Design Issues for Studies Enrolling Women

Common Problems

Much of the data obtained on the effects of the menstrual cycle on physiological systems are derived as adjuncts to other studies, and confounding variables are often not adequately controlled. Diet is seldom controlled, even in studies of serum lipids, blood pressure, or fluid dynamics. Activity is not always controlled in studies of energy intake; stresses are not controlled in studies of blood pressure. Techniques and procedures for sample collection, such as fasted or fed state, should be (but often are not) standardized. Sample size in many studies may be too small to identify significant effects of the hormone changes. Therefore, study results may have large variability as a result of uncontrolled factors that could potentially modify the outcome variable and may lead to inconsistent conclusions about menstrual cycle effects.

Many studies lack clear definition and objective identification of the phases of the menstrual cycle. Many rely on the subjects' self-report of the appearance of menstruation to number the days of the cycle and identify the phases. Investigators use different methods of numbering the days and identifying the phases of the cycle, making it difficult to compare studies. Some professionals count forward from

menstruation and designate the phases by convention. Some count backward to identify ovulation, using population averages for the length of the luteal phase. If the phases are not clearly identified in endocrinological terms, the timing of the hormonal changes and phases may not be precisely identified. The occurrence of folliculogenesis and ovulation should be confirmed if nonovulatory cycles are to be excluded from the analysis. Some otherwise well-characterized studies report data from only one cycle per subject. Investigators may not be blinded as to the phase of the subject.

In many studies that have attempted to control for the menstrual cycle, the follicular phase has been used exclusively because the hormone levels are steady and low. This may simplify study design but may obscure significant physiological effects that occur with the hormonal surges of ovulation and the luteal phase. Also, the length of the follicular phase varies more within and between women than does the luteal phase. To accurately study the effects of the hormonal cycle on physiological systems, the phases must be correctly identified and all phases should be studied.

Also to be considered in the design of research protocols, especially when cycle phase or hormonal status are outcome variables, are personal lifestyle factors, physiological characteristics, or environmental factors that can affect the cycle and contribute to unpredictability and to intra- and interpersonal variability, such as the following:

- Strenuous activity, inadequate nutrient intake, and mental and emotional stresses can alter the individual woman's cycle.
- Inadequate caloric intake, acute or long-term, attenuates hormonal rises and can prevent ovulation. Unnaturally low levels of body fat in women can prevent ovulation secondary to low hormone levels.
- Extreme physical exercise, with or without adequate energy intake, will prevent ovulation and disrupt the cycle (62). Modest exercise has not been shown to have this effect.
- Seasonal changes in the amount of daylight can also affect the cycle, especially in the far northern latitudes. Some women may experience ovarian hormone suppression during the dark photoperiod of autumn to spring.

The effects of vegetarian diets on menstrual cycle parameters and fertility, although of high interest, are not well understood. Pirke et al (63) reported that women who followed a vegetarian diet had an increased incidence of anovulatory cycles, with lower luteal-phase serum estrogen and progesterone levels compared to omnivorous women. Other investigators, however, found that behavioral factors associated with restrained eating habits, rather than vegetarianism per se, were related to menstrual irregularities (24). Different dietary levels of soy products, which contain estrogen-like compounds, also may confound the results of many studies on vegetarians (64). More well-controlled research is needed on this topic.

Improving Study Design

Statistical Power and Sample Size

When designers create the study protocol, a decision must be made whether a separate measure of the outcome effect is to be made in each status group (eg, males, females; males, premenopausal females, postmenopausal females; postmenopausal females with and without hormones) or whether data from all the groups will be combined for a single estimate of the effect. This may have enormous impact on statistical power and related sample size. If it is not necessary to make separate estimates, data from all subjects can be combined. To make separate estimates, sample size will roughly increase according to the number of groups. To be able to make a comparison of the effects in response to diet among the different groups, even more subjects must be enrolled.

Many studies will not have adequate power to actually compare the magnitude of response in men vs women. Rather there may instead be enough power to make separate statements of magnitude of response in men and in women. (Also see Chapter 2, ''Statistical Aspects of Controlled Diet Studies.'')

Hormonal and reproductive status can be considered as sources of between-subject or within-subject variation. Corresponding adjustments must be made in study design, statistical power calculations, data analysis procedures, and reporting of data.

The ovarian cycle contributes to within-subject variation, as phases change during a cycle and as cycles vary within the same woman, and also to between-subject variation among women. These sources of variation can be addressed at both the design and the analysis stages of the study. To control for cycle phase, the study's start date and specimen collection schedule may have to be tailored to each individual and may have to be kept flexible according to the events of each individual cycle (this may not be practical for larger studies).

The average cycle length of 28 days can be a useful consideration when investigators design studies and plan sample collections. Taking multiple outcome samples can control for within-cycle variation, and observations of cycle phase can be linked to other study results. For studies with relatively large numbers of participants, or for short-term studies ($<$1 month), it may be appropriate to assume that the subjects are evenly distributed across the phases of the cycle. This likely will control for the potential confounding because the diet intervention will be randomly started at different points in the subjects' cycles. Or, by monitoring and recording the cycle events during the study, this issue can be dealt with later during data analysis.

When a potentially important source of between-subject variation is known at the outset of a study, there are several ways to use the information to avoid confounding. Preferably, subjects can be classified according to status, and treatments then randomized for each status group. This will en-

sure that each treatment group contains a similar proportion of subjects from each status group. For example, if there are three diet treatment groups, randomization would place premenopausal and postmenopausal women into each diet group in numbers reflecting their proportions in the group as a whole. Alternatively, the study can be designed to recruit specific proportions of subjects in each status group. This process sometimes is referred to as *filling cells.* In either case, sample size calculations are used to determine the number of subjects required to provide sufficient statistical power to make separate estimates of each group's response to diet. If separate estimates cannot be made, data from all subjects are pooled to make a single estimate and then analyzed using statistical procedures that can ''adjust for'' status, ie, they incorporate weighting factors that reflect the proportions of subjects in each group.

In crossover designs, in which each subject receives all treatments and results are analyzed as within-subject comparisons, controlling for cycle phase becomes imperative. Results can be confounded if the endpoints from the different treatments are obtained in different phases of the cycle (eg, if Diet 1 endpoints were collected only during the luteal phase, and Diet 2 endpoints only during the follicular phase). By collecting endpoint data for each diet phase throughout the entire menstrual cycle, the investigator can detect the overall effect of diet without confounding. The same data will also allow the investigator to evaluate the effect of diet at each phase of the cycle (64, 65).

In parallel-arm protocols, in which subjects are randomized to one of multiple treatments, confounding can occur if many of the Group A subjects happen to be in, say, luteal phase, whereas many of the Group B subjects happen to be in follicular phase. If endpoint data are collected over the course of the entire cycle, or if cycle phase is at least monitored throughout the study, appropriate data analysis techniques can adjust for cycle phase during evaluation of the dietary treatment effects.

Data Collection and Analysis

The approximately 4-week length of the typical menstrual cycle should be considered when designing the collection of samples and data for study endpoints. If investigators expect that the menstrual cycle is likely to exert relatively strong effects on study results, it may be desirable to collect multiple endpoint samples or make multiple observations throughout the course of the cycle. Menstrual calendars or other data needed for staging the cycle are collected at the same time. It may be feasible to collect endpoint samples once weekly over a 4-week time period. The cycle phase for each subject can in this way be characterized adequately and then linked to the other study results.

For other studies, it will be necessary to take samples for endpoint measurements at precisely defined times of the cycle, such as Day 0. It also might be possible to collect endpoint samples at planned intervals, such as once per week during the last 4 weeks of an 8-week diet period, and use hormone levels or menstrual calendars to make a judgment about cycle phase. Few studies will have the financial resources to collect daily blood samples for hormone determinations, and subjects might become averse to repeated phlebotomies. Thus, a less intensive approach that yields adequate precision, albeit with slightly greater error (ovulation day (± 2 days instead of within a single day), may have advantages for many studies.

Data obtained to define cycle phase throughout the course of endpoint sample collection usually are formed into categorical or collapsed variables that lend themselves to stratified data analyses. For example, the information from a daily menstrual calendar (a sequence of categorical data points: menstruating yes/no) is often used to yield another categorical variable (such as menstrual phase: follicular or luteal). Blood hormone levels, although originally collected as continuous variables, also are often used in this way.

It is usually better, however, to take advantage of analysis techniques that incorporate ''uncollapsed'' continuous data without loss of detail. Such data can be included as covariates in multivariate models that adjust for potential confounding of study results by menstrual cycle phase. This approach generally has greater statistical power compared with stratified analyses, provided the data are in suitable form.

The sophisticated techniques that statisticians apply to circadian rhythm analyses (ie, patterns of diurnal and seasonal variations) also are suitable for analyses of the menstrual cycle per se (65, 66). Cyclic trigonometric models such as sine and cosine curves can be fitted to hormonal data and other continuous distributions, provided there are enough data points. This type of analysis might be useful, for example, in evaluating within-subject variation after stabilization on a constant diet. As mentioned earlier, least-squares methods can be turned to the analysis of basal body temperature data (24).

When menstrual cycle parameters are primary dependent outcome variables, the results are reported in the usual fashion with descriptive statistics. A good example is found in Cassidy et al (64). Direct dietary effects on the menstrual cycle, such as changes in phase length, hormone levels, and cycle length, can be assessed this way. Also, when researchers are studying the effects of exercise or light, for example, on the menstrual cycle, constant metabolic diets can be used to control for dietary confounding.

Women as Study Participants

Recruitment

Classification of women by hormone status must be unambiguous. Assigning a subject to the wrong category or having a subject change categories leads to loss of statistical power and errors in data interpretation, particularly if the investigator wishes to elucidate differences among groups. Appropriate advertisements and recruiting materials, along

with rigorously defined and carefully applied selection criteria, help the investigator to avoid recruiting subjects at risk for changing their hormonal status (such as entering menopause) during the study. The hormonal and reproductive status of women seeking to participate in a feeding study must be assessed in a systematic fashion directly linked to the study protocol. The numbers of subjects needed in a particular enrollment category and the exclusion criteria must be established as part of the study design.

For example, few investigators would enroll pregnant women unless the research question was specifically directed to them. Other investigators might seek to enroll a specific number of postmenopausal women but would not recruit those taking estrogens. Some protocols might require premenopausal females willing to keep detailed menstrual diaries but must exclude users of oral contraceptives.

Preliminary assessment of status is generally carried out using screening questionnaires administered over the telephone or during a clinic visit. Questions generally address whether the woman is pregnant or considering pregnancy; is lactating; has recently delivered a child or otherwise ended a pregnancy; is still menstruating or has attained natural or surgical menopause; and is taking oral contraceptives or postmenopausal hormonal preparations. For this last group, it is helpful to have the subject bring their pill packets with them to the clinic or to have various sample kinds of pill packets on hand at the clinic for identification to document accurately which formulation each woman is taking. Examples of screening questionnaires are found in Exhibit 8–2.

Eating disorders are particularly common among women (67) and should be screened for at the outset. Many people respond to recruiting advertisements for controlled feeding studies with the hope that they will lose weight. Unless weight loss is a planned study outcome, recruiters must be explicit that entry weight will be maintained. Screening questions about whether the prospective participant has had large weight fluctuations or has been dieting to lose weight will help the study team to identify individuals with these concerns.

Protection from Research Risks

Inclusion of women in research studies may require special considerations for informed consent statements and other institutional clearances. For example, women with regular menstrual cycles are more prone to anemia. This leads to higher dietary iron requirements but also puts a limit on the total volume of blood that can be drawn during the course of study.

Informed consent statements usually include commitments concerning participants' intention not to become pregnant and to inform the investigator immediately if pregnancy is suspected. Pregnancy testing may be necessary both initially and also periodically throughout the study if there is any potential risk to a developing fetus. Nutrition research generally does not usually pose risks to a fetus, but pregnancy may affect compliance with the protocol and confound interpretation of the study outcome parameters. If use of OC is an exclusion criterion, other reliable forms of birth control, such as tubal ligation, monogamous relationship with vasectomized partner, same-sex partner, or celibacy, are usually discussed with the subject before participant enrollment.

Well-controlled feeding studies occasionally are specifically conducted with pregnant or lactating subjects. Usually such studies are conducted to answer specific biologic questions about nutrient metabolism during these physiologic states. Under these circumstances, it is likely that the institutional review board (IRB) approving the protocol will require extra attention to safety issues and informed consent procedures. For example, use of radioactive isotopes is contraindicated in pregnant or lactating women unless specifically justified for these populations. Otherwise, enrolling a small number of pregnant or lactating women in a study addressing more general questions will introduce excessive variability into the study population. (See also Chapter 5, ''Ethical Considerations in Feeding Studies.'')

Participant Management Issues

Monitoring the menstrual cycle may be a new idea for many women recruited into studies. The activities may seem strange to them, especially if the study is not investigating a gynecologic topic. Education about the cycle and the importance of the data to the study is necessary to obtain good compliance, especially if self-monitoring procedures are being used. Privacy and confidentiality, as always, are of utmost importance. The clinic site must have an area with adequate privacy for reviewing menstrual calendars with subjects. Confidence and ease of the staff in discussing self-monitoring techniques in a straightforward manner will make the subjects more comfortable. The subjects should be emphatically reminded that monitoring the cycle does not, in itself, constitute contraception.

Financial reimbursement, even if small, helps to communicate the seriousness of the study and improves compliance and retention. An additional stipend for those women subjects who are monitoring their cycles is justifiable, especially to compensate for the burden of blood draws and the extra work required for recording the data.

Studying subjects in a specific phase of the cycle requires flexibility on the part of the staff and the facility. The subjects' schedules must be accommodated. If the subjects make telephone calls to give the investigators the results of self-monitoring techniques, it is helpful to establish codes so that subjects can call in the presence of others without being explicit. Usually communication between female staff and female subjects works best.

Halbreich et al (68) describe recruiting techniques for studies of menstrual cycle per se that can be used in studies to control for phase. A telephone checklist of inclusion and exclusion criteria can save time by avoiding unnecessary

EXHIBIT 8-2

Examples of Screening Questionnaires for Hormonal Status[1]

EXAMPLE 1: TELEPHONE SCREENING QUESTIONNAIRE FOR PREGNANCY AND LACTATION

WOMEN BORN AFTER 1953 ONLY

37. Are you pregnant or planning to become pregnant within the next year? YES NO

38. Are you breast-feeding? YES NO

39. Have you had a baby within the last 6 months? YES NO

If the answer to either question 37, or 38, or 39 is YES, then the applicant has become ineligible. If so, terminate the interview and complete questions 12 and 13.

EXAMPLE 2: CLINIC VISIT SCREENING QUESTIONNAIRE FOR HORMONAL STATUS

WOMEN ONLY

27a. Are you currently taking an oral contraceptive? YES NO

b. If YES to 27a, are you planning to stop? YES NO

c. If NO to 27a, are you planning to start? YES NO

[Circle the letter preceding the response.]

28. What is your current menstrual status?
    R Regular (normal) [go to question 30]
    I Irregular [go to question 29a]
    N Not menstruating [go to question 29c]

29. If you are menstruating irregularly, what is the reason?
    A Undergoing menopause
    B Other (describe): _____

30. If you are not menstruating, what is the reason?
    _____ Natural menopause
    _____ Hysterectomy
    _____ Medication stopped period
    _____ Other (describe): _____

31. When did you have your last period?
    A Less than 2 months ago
    B 2 months to 6 months ago
    C 6 months to 1 year ago
    D 1 year but less than 3 years ago
    E At least 3 years ago

32. a. Are you taking or have you ever taken estrogen? [Estrogen or female hormones for hot flashes or symptoms of menopause] YES NO

    b. If YES to 31a, are you currently taking estrogen? YES NO

    c. If NO to 31a, do you plan to start taking estrogen? YES NO

Resume asking questions of all applicants.

[1]Adapted from *Manual of Operations for the DELTA Program* (74).

screening appointments, but face-to-face contact is required to establish and build rapport. Continued contact by a specific staff member and generation of a ''group'' feeling among the subjects improves recruitment, compliance, and retention. Detailed screening procedures that correctly classify subjects are worthwhile.

As mentioned earlier, serious eating disorders and excessive concerns about body weight are prevalent in the general population of US women (67). Good screening questionnaires and interviews will likely eliminate individuals with clinical-grade eating disorders. Milder forms of these problems, however, may surface during the protocol, affecting compliance and ability to complete the study. For example, female participants may be more concerned than are males during the study about body weight fluctuations, energy content and cognitive attributes of study foods, and minor gastrointestinal and other physical side effects of the research diet. Investigators should be prepared to identify and address these issues appropriately.

The collection of biological samples for study endpoints may be affected by reproductive status or menstrual cycle phase. For example, as mentioned earlier, the total volume of blood that can be drawn from premenopausal women may be constrained by risk of anemia. Another example is that of urine collection; contamination by menstrual blood may affect some assays and special collection procedures may be needed.

Planning Research Diets

For studies enrolling both males and females, nutritional adequacy is always a primary consideration, but there generally are no major gender effects on diet planning. However, investigators must recognize that smaller women and older women, particularly after menopause, may have fairly low energy needs. B-vitamin requirements, such as thiamin, riboflavin, and niacin, are tied closely to lean body mass and more broadly to energy intake and are not especially difficult to provide.

It can be difficult, however, to achieve nutrient goals for dietary components that are needed as absolute total daily levels rather than relative amounts (ie, per 1,000 kcal). For example, a fiber allowance of 15 g/day is difficult to achieve on a 1,500 kcal diet; iron, zinc, and folate present a similar challenge. This reflects the generally higher nutrient density diets that women need. The Food and Nutrition Board has acknowledged that women have different nutritional needs at different life stages (69–71): women require less iron after menopause and more folate during pregnancy, for example. Reaching the new calcium goals of 1,000–1,500 mg/day (72) can be particularly problematic, depending on study design and the particulars of the research diet; fortified foods, such as calcium-supplemented orange juice, may be necessary.

Fixed nutrient levels specified by study design can be difficult to provide when the study participants have a large range of energy needs. In the DELTA studies that enrolled males and females consuming 1,500–3,000 kcal/day, a single level of total daily cholesterol (300 mg/day) was provided to all subjects regardless of calorie intake by adding egg yolk powder in varying amounts to each calorie level (73, 74). Otherwise, if all foods had been increased or decreased in proportional increments, the higher calorie diets would have contained far more cholesterol.

If pregnant or lactating women are enrolled in controlled feeding studies, researchers are ethically obligated to ensure that the diets meet their increased nutrient needs. Folate and mineral requirements are especially elevated (69). Menus should not include foods generally known to be unappealing to pregnant women, who are prone to taste and smell aversions. For lactating women, menus should be reviewed for foods that might be considered to predispose the infant to colic (eg, cabbage) or which might make the milk taste unpleasant to the infant (eg, garlic).

Conclusion

Controlled diet studies provide the most fundamental way to obtain data on nutrient requirements for women of all ages. By eliminating some potential sources of confounding, controlled feeding studies can also be a good way to clarify various aspects of female reproductive biology. Moreover, even if there is no major expected effect on study outcome variables, accounting for hormone status and the menstrual cycle in the study design may enhance the precision of the research. By paying more attention to protocol design, data collection techniques, hormonal status definition, and data analysis, the quality of research conducted with female subjects can be greatly improved. The research techniques for addressing these points are feasible and often inexpensive and should serve to promote the inclusion of women in nearly all study protocols.

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