

## CLINICAL STUDY

## Diurnal rhythm of free estradiol during the menstrual cycle

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### Abstract

**Objective:** To investigate the diurnal rhythm of estrogens in normally cyclic women during reproductive life.

**Design:** Multiple saliva sampling in normally cyclic healthy women during reproductive life at different phases of their menstrual cycles was carried out.

**Methods:** Salivary estradiol was measured by radioimmunoassay in samples collected every 2 h for 24 h from 15 normally cyclic healthy women during reproductive life during the menstrual phase, the late follicular/peri-ovulation phase, the early to mid luteal phase and the late luteal phase, respectively, of their menstrual cycles. The levels of salivary estradiol were analyzed by means of periodic regression.

**Results:** A daily biological rhythm of free estradiol was found after quantification with a nonlinear periodic regression model. The observed diurnal free estradiol rhythm consists of two major components: an asymmetrically peaked diurnal cycle and ultradian harmonics in the range of 6 to 12 h. The diurnal and ultradian rhythms were remarkably consistent throughout the menstrual cycle in terms of mesor (24 h mean level), peak width and amplitude. There was a tendency for the 24-h rhythm acrophases to converge in the early morning, while the acrophase of the menstrual phase occurred significantly later than in the late follicular/peri-ovulation phase.

**Conclusions:** The diurnal rhythm of estradiol has a similar complex temporal organization for different menstrual phases. The menstrual cycle mainly modulates the acrophase of the diurnal rhythm.

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### Introduction

It is known that estrogens are more than just 'sex hormones' that play a principal role in regulating behavioral and physiological events essential for successful procreation (1). Estrogens are also important for the protection of the cardiovascular system (2) and for the maintenance of bone (3, 4). Estrogens play an important role in the sexual differentiation of the brain (5) and in modulating the regulation of the autonomic and reproductive neuroendocrine system, mood, and cognition (5–7). Changes in estrogen level in different physiological and pathological situations are a topic of considerable research interest. Previous studies on estrogen levels mainly focused on their fluctuation during the menstrual cycle, postpartum and menopause, using single or multiple time-point samples (8–10). Although studies on estrogen secretion in early puberty, gestation and breast cancer have assumed for a long time that estrogen levels in humans have a diurnal

rhythm (11–15), little is known about the fluctuation of estrogens in normally cyclic women over the 24-h period, i.e. its daily biological rhythm. Knowledge of the daily secretion profiles of estrogen will contribute not only to our understanding of its function but also offer useful reference data for further study on the roles of rhythms in estrogen secretion in various diseases.

It is known that both the hypothalamic gonadotropin-releasing hormone (GnRH), and the pituitary luteinizing and follicle-stimulating hormones have pulsatile secretion profiles (11–16) and that these hormones affect the secretion of estrogens within the human hypothalamic–pituitary–gonadal axis. It is suggested in studies with both animal and human material that the hypothalamic suprachiasmatic nucleus (SCN), the endogenous circadian pacemaker in the brain, is sex hormone sensitive and is involved in the generation and regulation of the female reproductive cycle (17, 18). Based on the observations mentioned above,

it is reasonable to hypothesize that the biological rhythm of estrogens is composed of a complex of cycles including not only the monthly cycle but also circadian rhythms and ultradian pulses. The recent development of non-invasive saliva measurements of steroid hormones (19) allowed us to investigate the diurnal and ultradian rhythms of salivary free estradiol, i.e. the biologically active form of estrogen, in women of reproductive age in their natural environment. We further explored whether circadian and ultradian rhythms of free estradiol are modulated by the menstrual cycle.

## Materials and methods

### Participants

Fifteen healthy Chinese women (mean age 32.8 years, range 25–40 years) entered the study between 25 March and 20 June 2001. They all took part on a voluntary basis, and had regular menstrual cycles with periods from 27 to 30 days. Exclusion criteria were as follows: abnormal body mass index (BMI  $\leq 18$  or  $\geq 25$ ), medication for chronic illnesses, oral contraceptives within the last 6 months, lactating within the last 12 months, evidence of infertility, or presence of sexually transmitted diseases. All subjects signed a consent form and the study was approved by the Anhui Medical University review board.

### Procedures

Subjects participated in the study for a total of four days over a period of one month, based upon their self-reported menstrual cycle day-count: phase I, one day from day 2–3 (menstrual phase); phase II, one day from day 12–14 (late follicular, peri-ovulation phase); phase III, one day from day 18–23 (early to mid luteal phase); phase IV, one day from day 25–28 (late luteal phase). In phase II, ovulation was determined by changes in body basal temperature and every subject had an ovary ultrasonic examination in the B mode to help ensure the phase according to the dimension of the dominant follicle and to ensure the presence of a normal genital system (20). At the entrance time of each day, they were given a package containing 12 Salivettes (Sarstedt, Nümbrecht, Germany) (21) and were asked to collect saliva every 2 h from 0800 h to 0600 h the next morning. Between 2200 h and 0600 h the next morning the subjects went to bed/got up with the light(s) turned off/on respectively. This schedule was not significantly different from their work and rest routine. Saliva samples were collected with small polyester tampons held in the mouth for about 5 min to soak up the saliva and were then placed in the Salivette container. The Salivettes were sealed and stored at 4 °C in a refrigerator immediately after collection of the samples during the daytime and placed in a cold box beside

the subject's pillow at night until the end of each 24-h period. The samples were centrifuged for 10 min at 3000 *g* and frozen at –20 °C until assayed. The subjects were non-heavy exercisers, non-alcohol/non-caffeine drinkers and non-smokers. Subjects were studied in their habitual environment with their habitual occupations. They were advised to brush their teeth without toothpaste, to drink plenty of water but to avoid eating and to rinse their mouths thoroughly with water 30 min before sampling during the daytime. They were also asked to avoid sexual intercourse during the sampling day. A total of 57 sets of data points were gathered in the present study (no data were available for phase I of subject #10 and phases I and IV of subject #8 because of the lack of sufficient saliva at some time-points). Please note that we did a pilot study before the formal study with two subjects for two days, every other day of phase II (late follicular, peri-ovulation phase) respectively, in which the saliva sampling interval was set at 1 h between 0800 h and 2200 h and at 2 h between 2200 h and 0600 h the next morning. The study of the secretion pattern of estradiol in the pilot study suggested that a 2-h sampling interval throughout the day (which is more convenient for participants) would not affect the deduced diurnal rhythm pattern, which is the main topic of our study.

### Salivary estradiol assay

Salivary estradiol ( $E_2$ ) concentrations were measured with the use of a double-antibody radioimmunoassay (RIA) with  $^{125}I$ -labeled  $E_2$ . Antibody and tracers were derived from DSL-39100 estradiol (third generation; Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The estradiol antibody is rabbit anti-estradiol in a protein-based buffer with sodium azide. The antibody cross-reacts 6.9% with estrone and less than 1.0% with  $17\beta$ -estradiol-3-glucuronide. The protocol used was that according to Shirtcliff *et al.* (22). The standard curve of the salivary estradiol assay was highly reproducible, with an average correlation coefficient of 0.9969 in this study. The average intra- and interassay coefficients of variation were 7.2 and 9.9% respectively. Analytical recovery was on average 100.7%. Linearity was assessed (1:2 to 1:12 dilutions) across the range of measurements with an average recovery of 89% (range 76%–103%). The range of standards was 0.375–7.500 pg/ml. The analytic sensitivity was 0.250 pg/ml.

### Statistical analysis

Examination of the raw data indicated that both ultradian and diurnal components were clearly present, with amplitudes of comparable magnitude. Given the variance resulting from such ultradian periods, fitting just a plain 24-h periodic function (cosinor, see reference 23) will only occasionally result in a fit that is

significantly better than the single parameter model of just a mean with residual error. We used Rao's F-distributed T-statistic (24), which applies a penalty for increasing numbers of parameters, to evaluate several multi-harmonic models and to select the best and most parsimonious model. Applying Nyquist's rule, our sampling frequency allowed for investigation of ultradian rhythms with periods of 5 h or more. Moreover, skewness or peakedness of circadian physiology is the rule rather than the exception, e.g. the 8 h to 16 h asymmetric sleep-wake cycle. Such asymmetric phenomena are by far the most parsimoniously modeled by the peaked and skewed functions proposed by Batschelet (25). Curve fitting was performed using constrained nonlinear regression analysis (SPSS Release 10.0).

After selection of the best model, the possibility of modulation of the parameters by the phase of the menstrual cycle was examined using repeated measures ANOVAs, and circular statistics in the case of parameters representing the acrophase (Jupp's Phi and S for mean angle and angular standard deviation, Mardia-Watson-Wheeler's Chi squared test for evaluation of acrophase differences over the menstrual cycle) (25).

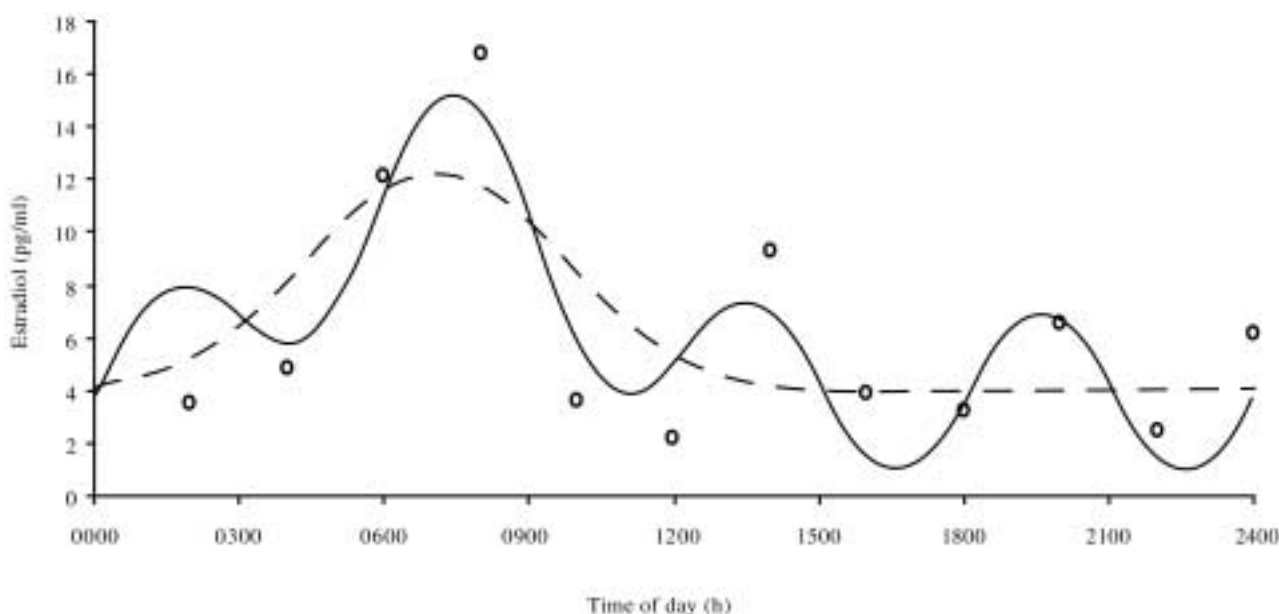
## Results

The best-fitting model describing the rhythmic phenomena, though still parsimonious, was selected from a

series of peaked and skewed cosine functions with the addition of a single higher order harmonic, i.e. the 2nd, 3rd or 4th harmonic (24). The most adequate model turned out to be a peaked cosine function with one additional harmonic, which was significant at  $P < 0.05$  or better in 42 cases, and at  $P < 0.10$  or better in 53 cases:

$$y[t] = M + A * \text{COS}[(t - \Phi_1) + v * \text{SIN}(t - \Phi_1)] + B * \text{COS}(u * t - \Phi_2)$$

where  $t$  represents the time of day (in radians),  $y$  the predicted value of estradiol at time  $t$  and  $M$  the mesor (24-h mean level). A correction to the mesor is applied in order to account for the asymmetry of the rhythm (25).  $A$  is the amplitude of the diurnal component,  $\Phi_1$  its phase angle and  $v$  its parameter of 'peakedness'. The peakedness parameter can be transformed to represent duration in hours (25).  $B$  represents the amplitude of the ultradian component,  $\Phi_2$  its phase angle and  $u$  the harmonic of the ultradian component, where  $u = 2, 3$  or  $4$  correspond with rhythms of 12, 8 or 6 h. The acrophase is the corresponding time-point of the predicted highest value of  $y[t]$ . Figure 1 shows an example (subject #11, phase III) of raw data and the fitted model. All parameters measured over the four phases for which the significance level of the fitted curve was less than 0.10 were used for further calculation of parameter means and examination of modulation of the rhythm throughout the menstrual cycle.



**Figure 1** Representative example (subject no.11, phase III) of raw data points (circles), the peaked cosine 24-h rhythmic component (dashed line) and full model including a 4th harmonic (solid line). Note the asymmetry between the peak (0612h) and trough (1748h) duration and the comparable magnitude of the diurnal (4.2 pg/ml) and ultradian (3.0 pg/ml) amplitudes. The diurnal acrophase occurred at 0702 h in the morning. The model accounted for 73% of the estradiol variance, and was statistically significant ( $F(1, 6) = 16.35$ ,  $P < 0.01$ ).

The rhythms of free estradiol are remarkably robust throughout the menstrual cycle. With the exception of the acrophase, none of the diurnal and ultradian parameters were affected by the phase of the menstrual cycle. Table 1 shows the means and standard deviations of all relevant parameters, where the mesor and peakedness are expressed in their corrected format (25). The mesors and the amplitudes of the circadian and ultradian components of free estradiol in the menstrual (phase I) and late luteal phases (phase IV) were slightly, but not significantly, lower than those in the late follicular/peri-ovulation (phase II) and early to mid luteal (phase III) phases. The acrophase, however, occurred later in phase I (menstrual phase) of the menstrual cycle. The Mardia-Watson-Wheeler test indicated a significant difference between the acrophase in menstrual phases I and II (Chi squared test = 6.66, degrees of freedom (df) = 2,  $P < 0.05$ ), and a trend towards a difference between the acrophase in menstrual phases I and IV (Chi squared test = 4.92, df = 2,  $P < 0.10$ ). Also, acrophases were found to be concentrated in the early morning (Fig. 2). The most frequently occurring ultradian component, in 56% of the cases, was the 4th harmonic, corresponding to a period of 6 h. However, in 23% of the cases the ultradian rhythm was better described by the third harmonic, corresponding to a period of 8 h, and in 21% of the cases by the second harmonic, corresponding to a period of 12 h.

## Discussion

The present results indicate that salivary estradiol in women of reproductive age has a nonlinear periodic rhythm throughout the menstrual cycle. This rhythm contains diurnal and ultradian components of similar magnitude. The diurnal component is asymmetrical, i.e. the duration of the peak differs markedly from that of the trough. There is considerable variation among subjects in the ratio between peak and trough duration: both peaked and flat-topped rhythms occur. The rhythms are remarkably consistent throughout

the menstrual cycle. The menstrual cycle mainly modulated the acrophase of the estradiol rhythm, indicating that during the menstrual phase the peak levels occurred later in the morning.

There are several protocols designed to study a daily biological rhythm, such as the free-run, the forced desynchrony and the constant-routine protocol (26, 27), yet the control methods might not reveal all factors of importance for the biological rhythms of hormones in women. For example, a group of women living together in a room might have 'menstrual synchrony' (28), while synchronization of life style might result in obvious changes in the mood and even in the rhythm of healthy women. In the present study, the subjects were living their normal lives and were tested in the same season and with fixed bedtimes and get-up times, allowing us to investigate the diurnal rhythms of hormones in females living under natural conditions.

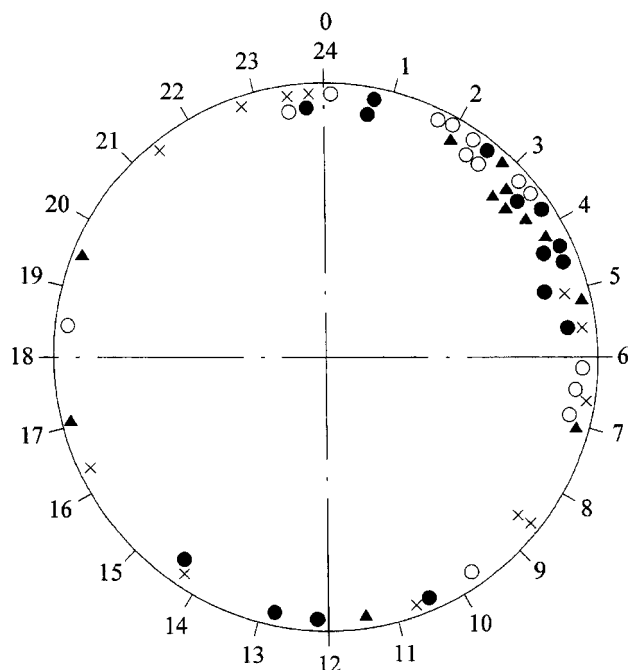
Many clinical investigations have demonstrated that salivary steroid measurements are a practical and convenient approach to assess fluctuations in diurnal rhythms. Salivary levels of estradiol are believed to represent accurately the 'free', i.e. the biologically active, fraction in the general circulation (19, 29). It has been shown that unconjugated steroids enter the saliva by diffusing through the cells of the salivary glands and that their concentration in saliva does not depend on the rate of saliva production (30). The levels of salivary estradiol in the present study were largely comparable to those in previous studies measured during different menstrual phases (31, 32) and as measured in blood samples (20). The difference in free estradiol mesors between the menstrual phases, however, was not statistically significant. This might, on the one hand, be due to between-subject variability of the peak time within the menstrual cycle: both the mesor and the amplitude were elevated in phases II and III as compared with phases I and IV. Moreover, it may be that the previously reported increase in estradiol in phases II and III might be more pronounced in bound estradiol, whereas in the present study we assessed free

**Table 1** Characteristics of the diurnal and ultradian rhythms in estradiol throughout the four phases of the menstrual cycle. Results are means  $\pm$  standard deviations, but the acrophases are calculated as angular means and standard deviations. The unit for mesor and amplitude is pg/ml and the unit for acrophase and peak width is h and min. The ultradian harmonic row gives the mean and standard deviations of the best fitting harmonic number (of 2, 3 or 4).

Parameter	Phase I	Phase II	Phase III	Phase IV
True mesor	4.93 $\pm$ 1.27	6.07 $\pm$ 2.45	5.79 $\pm$ 2.46	4.97 $\pm$ 2.60
24-h amplitude	3.14 $\pm$ 1.60	5.15 $\pm$ 4.22	4.66 $\pm$ 3.18	4.01 $\pm$ 3.71
Acrophase	0822 $\pm$ 0454*#	0246 $\pm$ 0306	0547 $\pm$ 0400	0346 $\pm$ 0338
Peak width	1312 $\pm$ 0545	1131 $\pm$ 0516	1355 $\pm$ 0502	1228 $\pm$ 0502
Ultradian amplitude	1.99 $\pm$ 1.08	2.37 $\pm$ 2.20	3.00 $\pm$ 2.13	2.05 $\pm$ 1.37
Ultradian harmonic	3.54 $\pm$ 0.78	3.40 $\pm$ 0.83	3.33 $\pm$ 0.72	3.14 $\pm$ 0.95

\*  $P < 0.05$  compared with phase II, Mardia-Watson-Wheeler  $\chi^2 = 6.66$ , df = 2.

#  $P < 0.10$  compared with phase IV, Mardia-Watson-Wheeler  $\chi^2 = 4.92$ , df = 2.



**Figure 2** Distribution of acrophase values among different menstrual phases in a total of 53 women. Phase I (x), menstrual phase ( $n = 12$ ); phase II (O), late follicular/peri-ovulation phase ( $n = 14$ ); phase III (●), early to mid-luteal phase ( $n = 15$ ); phase IV (▲), late luteal phase ( $n = 12$ ). Note acrophases were concentrated in the early morning.

estradiol, estimated to represent only about 1–2% of the total estradiol (33). Our finding that acrophases converge in the early morning is in agreement with the studies of Mitamura *et al.* (12) and Norjavaara *et al.* (34) on diurnal rhythms of estradiol. They applied a 24-h blood sampling protocol in children before and during puberty. It was proposed that the increased secretion of estradiol in the early morning was associated with the nocturnal rise in gonadotropin secretion (34). Our finding of a delay in the morning peak time in the menstrual phase deserves further study.

Animal studies have shown that the SCN is the endogenous circadian pacemaker in the brain which plays an important role in the control of hormonal biological rhythms (17, 35). Recent studies have shown that the human SCN expresses receptors for estrogen, androgen and progesterone (18, 36). Thus, although a peripheral circadian pacemaker rhythmicity, e.g. one originating in the ovaries, cannot be excluded, the diurnal rhythm of estrogens, as found in the present study, is most likely driven by the SCN, and is integrated with signals from the hypothalamic GnRH and the pituitary gonadotropin secretion profiles (11–16).

The study of estrogen requires full appreciation of its oscillatory dynamics, and the value of evaluating a person's estrogen levels based on a single or just a few time-points is quite limited.

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