The Salivary Ferning Test and Ovulation in Clomiphene Citrate-Stimulated Cycles

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Objective: To determine the day of ovulation by the salivary ferning test in clomiphene citrate-treated women.

Design: A descriptive study.
Setting: Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
Subject: Seventy-five infertile women with regular menstrual cycles.
Material and Method: Infertile women were given 100 mg of clomiphene citrate for five days and collected their saliva samples daily until seven days after ovulation. Transvaginal ultrasound was performed daily to detect ovulation. The salivary ferning formation was examined by a normal light microscope and graded from 1-3, according to its extent and intensity.
Main Outcome Measure: The salivary ferning score, the peak salivary ferning day, and the day of ovulation detected by ultrasound.
Results: The patients' age and cycle length (mean ± SD) were 32.9 ± 3.7 years and 28.4 ± 1.3 days. The peak salivary ferning day corresponded with the ultrasound ovulation day in only 7.1%. There were two peaks of median salivary ferning scores; one was two days prior ovulation and the other was five days post ovulation. There was no correlation between the peak salivary ferning day and day of ovulation detected by ultrasound (r = 0.102, p > 0.05).
Conclusion: In clomiphene citrate-stimulated cycles, the saliva ferning test does not seem to associate with ovulation.

Keywords: Clomiphene citrate, Saliva, Ferning, Ovulation

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There is an increasing demand for cheap self-tests to predict the fertile period in each menstrual cycle. Monitoring ovarian function by basal body temperature (BBT) or cervical mucus has been helpful but often misleading(1-3). Although the urine luteinizing-hormone (LH) test seems to be an effective predictor of ovulation, cheaper and simpler methods have been introduced(4,5). In recent years, a variety of small hand-held microscopes have been developed and marketed for the purpose of self-observing ferning patterns in saliva. Theoretically, the ferning (crystallization) pattern of saliva coincides with the female fertile period. The ferning is caused by NaCl, which cyclically increases under the influence of estrogen(6,7).

In natural menstrual cycles, the correlation between salivary ferning and the fertile period were reported(8,9). A recent study compared the salivary
ferning test and the self-detection of LH in the urine. They found that the peak in the salivary ferning fell within ±3 days of the LH surge in 76.2% of cycles and there was a highly significant correlation between the LH surge in urine and the peak day of salivary ferning\(^{(10)}\). Salivary ferning may be used as a new parameter to aid women to detect their fertile period.

Later, there was an increase in the usage of salivary ferning test in different clinical settings such as to predict the ovulatory period in clomiphene citrate-treated cycles\(^{(11)}\), to detect the resumption of ovarian function after extended breast feeding in the postpartum period\(^{(12)}\). Therefore, further research should be conducted to improve the use of the salivary ferning test in special situations.

Clomiphene citrate (CC) is the most common ovulation induction agent to be used in clinical practice. It is a non-steroidal triphenylethylene derivative. It has both estrogenic and anti-estrogenic properties\(^{(13)}\). Whether the salivary ferning test can determine the fertile period or ovulation time in patients treated with CC, which has anti-estrogenic properties, is still in doubt. There are evidences suggesting that estrogens have a biological role in salivary glands, but the expression of estrogen receptors within these tissues is an area of controversy\(^{(14,15)}\). The prior study\(^{(11)}\) conducted in 10 CC-induced cycles, found that in six of ten cases, the maximum degree of salivary ferning coincided with the day of ovulation determined by ultrasound, while the remaining four cases were obtained two days before or after ovulation. A larger number of cycles will need to be studied to confirm this preliminary finding and provide further understanding of salivary ferning in CC-treated cycles.

The purpose of the present study was to determine the day of ovulation by the salivary ferning test in CC-stimulated cycles.

### Material and Method

#### Subjects

Ninety women were recruited from March 2004 to January 2005 in the present study. The patients’ age ranged from 20-40 years and should have regular menstrual cycles of 24-35 days interval. All the patients had indication for induction of ovulation with CC. Women who had used hormonal derivatives within 3 months, had a diagnosis of polycystic ovarian syndrome, had a diagnosis of xerostomia\(^{(16-18)}\), smoking\(^{(19)}\), had infection/inflammation in the oral cavity\(^{(20,21)}\) or had ovarian cysts detected on ultrasound day 2 of their cycles were excluded from the present study. All women gave informed consent after counseling and the present study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

#### Study design

The present study is a descriptive study. All subjects underwent transvaginal ultrasound (TVS) (Aloka, Model SSD-1700; Aloka Co., Ltd., Tokyo, Japan) on day 2 of the cycle to rule out ovarian cysts. Subjects with a satisfactory ultrasound examination have 100 mg of CC (Clomid; Merrell Pharmaceuticals Inc., Kansas City, KS) from day 3 to day 7 for ovulation induction and were instructed about the method to collect their saliva samples daily, starting on day 3 of the cycle to 7 days after ovulation.

TVS was performed on the 10th day of the cycle to monitor the follicular growth and endometrial thickness. If one or more leading follicles with ≥18 mm in diameter were detected, hCG 5000 IU (Pregnyl, N.V. Organon, OSS, the Netherlands) was injected intra-muscularly to trigger ovulation. Single intrauterine insemination was performed 36 to 40 hours later. The ovulation was confirmed by ultrasound examination, if not, TVS was performed daily until evidence of ovulation appeared. Diagnosis of ovulation was made according to the usual sonographic criteria namely shrinkage or disappearance of the follicles and/or accumulation of free fluid in the pouch of Douglas\(^{(22,23)}\). The day of ovulation was considered day OV and the preceding days as day -1, -2, -3 and so on. Serum estradiol levels \((E_2)\) were evaluated on the day of ovulation and 7 days after that. All subjects were followed up two weeks after insemination to check serum beta-hCG level. Pregnancy was determined by the more than 50 mIU/ml of serum beta-hCG level.

#### Salivary ferning

The saliva sample was collected daily before the meal in the morning, using a clean dry finger placed sublingually. The tip of the tongue must be pressed to the palate. A drop of non-foamy saliva was placed on a glass slide and left to dry at room temperature for 3-5 minutes and subsequently examined by a 100 x normal light microscope (Olympus). The arborization is characterized by main stems among the leaves with side-branches showing tooth like projection. The fern formation was graded from 1-3\(^{(10)}\), according to its extent and intensity as shown in Fig. 1, with 1 = no visible ferning (random and unconnected dots), 2 = partial ferning (a combination of dots and ferns), 3 =

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full or peak ferning (complete fernlike patterns). The entire area of each saliva samples on glass slides was examined. In each visual field, the fern formation was scored. The final salivary ferning score was reported based on the best grading of fern formation. All of the slides were examined by one observer (S.P.). For the present research study, the peak salivary ferning was considered at the first day of the highest rated ferning pattern.

 Serum E₂, FSH and LH concentrations
Venous blood samples were allowed to clot and the separated samples were stored at -20°C until assayed for E₂, FSH, and LH level. All serum samples were assayed simultaneously for each hormone, using a fluoro-immunoassay (DELFIA; Wallac Oy, Turku, Finland). The intra-assay and inter-assay coefficients of variation were 2.39% and 3.08% for the E₂ level, 2.71% and 5.29% for the FSH level, and 3.60% and 4.52% for the LH level, respectively. The lower limits of detection were 13.6 pg/ml for the E₂ level, 0.05 IU/L for the FSH level, and 0.05 IU/L for the LH level.

 Statistical analysis
Data analysis was performed using the Statistical Package for the Social Sciences for Windows, Release 11.0 (SPSS Inc., Chicago, IL). Demographic data were calculated in percentage or mean (standard deviation: SD). Spearman Rank Correlation Coefficient was used to determine relationships between the day of ovulation detected by TVS and the day of peak salivary ferning. A p-value < 0.05 was considered statistically significant.

 Results
Ninety women were included in the present study. Their mean (+ SD) age was 32.9 ± 3.7 years (range, 20-40 years). Their mean (+ SD) body mass index was 20.8 ± 2.1 kg/m² (range, 17.3-29.7 kg/m²). Their mean (+ SD) cycle length was 28.4 ± 1.3 days (range, 25-34 days). Ten women were lost to follow up. Three women had no follicular growth after CC induction. One woman developed oral ulcer during the treatment and another one had over response from CC stimulation. Therefore, 75 women could be studied and they generated 98 CC-induced cycles. Their cause of infertility and basal hormonal level on day 2 of menstrual cycles were reported in Table 1.

The distribution of peak salivary ferning, according to the day of cycle regarding the ovulatory day is shown in Table 2. In 7 cycles (7.1%), the peak salivary ferning day coincided with the day of ovulation detected by TVS. In 36 of the 98 cycles (36.7%) the peak in salivary ferning fell within ± 3 days of the ovulatory day.

There was no correlation between the day of peak salivary ferning and day of ovulation detected by TVS (r = 0.102, p > 0.05) (Table 3).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>32.9 ± 3.7*</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>20.8 ± 2.1*</td>
</tr>
<tr>
<td>Duration of infertile (years)</td>
<td>5.1 ± 3.4*</td>
</tr>
<tr>
<td>Cycle length (days)</td>
<td>28.4 ± 1.3*</td>
</tr>
<tr>
<td>Causes of treatment (n, %) (98 cycles)</td>
<td>62 (63.3%)</td>
</tr>
<tr>
<td>Unexplained infertility</td>
<td></td>
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<tr>
<td>Pelvic endometriosis</td>
<td>36 (36.7%)</td>
</tr>
<tr>
<td>Male factor</td>
<td>10 (10.2%)</td>
</tr>
<tr>
<td>Basal hormone levels</td>
<td></td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>6.6 ± 1.9*</td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>4.4 ± 1.5*</td>
</tr>
<tr>
<td>E₂ (pg/ml)</td>
<td>41.6 ± 14.9*</td>
</tr>
</tbody>
</table>

* mean ± SD
The distribution of serum E₂ concentrations according to salivary ferning scores at day of ovulation and 7 days post ovulation are shown in Fig. 2 and 3. The mean ($\pm$ SD) E₂ on the day of ovulation and 7 days after ovulation were 222.9 $\pm$ 154.4 pg/ml and 479.2 $\pm$ 319.3 pg/ml, respectively. There was no correlation between serum E₂ concentrations with saliva ferning score on both days. Twelve women were pregnant (pregnancy rate = 12.2%). Eight were singleton pregnancies, two were twin pregnancy, and two were extrauterine pregnancy.

**Discussion**

During the past several years, researches have shown that various tests using saliva can also assist women to timing ovulation. Some of these tests
are based on electrolyte levels or the use of ELISA tests or other chemical based tests to determine the hormone levels in the saliva. This is then used to predict ovulation. It has long been known that the reproductive cycle in the women is regulated by hormones, and that these hormones are present in blood as well as in other body fluids. Human saliva was a very good source of both hormones and enzymes and their levels changed in accordance with the menstrual cycle.

Saliva in human beings is produced mainly in the parotid, sublingual, and submaxillary glands. Saliva samples are easy to obtain without trauma and can be obtained on a daily basis. It is known that saliva ferning depends principally on the electrolytes concentration (especially NaCl) and chemo-physical properties of the mucins it contains (sialic acid). The estrogens increase the water content in mid-cycle and determine the most favorable condition, optimal proportion of water, and optimal amounts of salts and sialomucin. It was claimed that there were many advantages of salivary ferning test such as its high accuracy, low cost, convenience, re-usable and easy to use.

Smith, et al found that for initial attempts at ovulation induction with CC in unselected patients, high technology monitoring offers no advantage over low technology monitoring in improving cycle fecundity or overall conception rates during the first 4 cycles of therapy.

Fedele et al studied 15 infertile patients for 25 CC-treated cycles to compare the predictive value of the basal body temperature (BBT), cervical mucus, serum LH and urinary LH in timing ovulation. BBT had low value as a predictive test for the time of ovulation, whereas cervical mucus had a predictive value of 59%, serum LH 63.6% and urinary LH 63.6%. The urinary LH was as reliable as serum LH measurements and has greater practical advantages. Because of the wide use of the salivary ferning test to predict non-fertile periods in natural family planning program, the authors wanted to know the data of this test in timing ovulation in CC-induced cycles.

In the present study, the day of ovulation as determined by saliva ferning score corresponded to the transvaginal ultrasound in only 7.1% of cycles. In 36.7% of cases, the peak salivary ferning response occurred within three days, before or after, of ovulation detected by TVS. These findings were in contrast to the results of a previous report that studied normal menstruation volunteers. In Fehring et al study they reported 76.2% of cycles that the peak salivary ferning
coincided within ± 3 days of LH surge. Another study was conducted to evaluate the efficacy in ovulation detection methods used in natural family planning in comparison with pelvic ultrasonography. They found that the salivary ferning test had a 36.8% ovulation-detection rate, but 58.7% of the results were uninterpretable(5).

Braat et al(33) reported the use of salivary ferning test to predict ovulation in 30 women with regular menstrual cycles. The sensitivity and the specificity of the test were 53% and 72% respectively. They also compared E2 concentrations for 31 saliva samples with serum E2 values. There was a strong correlation between saliva and serum, but no correlation between the E2 concentrations in saliva and the ferning aspect. In the same way, the present study found that there was no correlation between serum E2 concentrations and salivary ferning in both ovulatory day and seven days after ovulation.

In many cycles, ferning days were found throughout the cycle, and in other cycles, ferning was found only on 1 or 2 days. There was also no discernible beginning or end to the fertility cycle as determined by the salivary ferning test. Most of the records did not show the typical pattern that was reported in the literature(11), but they frequently showed a monophasic pattern. These may be due to the significantly higher serum E2 levels on all days of the pre-ovulatory phase of CC-induced cycles compared to spontaneous cycles(23) and the inconstant estrogen dilution in the saliva, yielding different patterns between women and within the same subject between cycles. Although subjects were given verbal and written instructions on how to place the saliva on the slides, there could have been individual variations in the collection process. Both ferning and non-ferning features can be observed in the same sample of dried saliva when viewed through a normal microscope with a wide field of vision.

The present study had some limitations that could not control confounding factors that interfered with the ferning process(34) (temperature, food and beverages), then it might not apply for clinical use. One of the explanations why there was no correlation between ferning effects and ovulation period was the anti-estrogenic properties of CC in the salivary glands(35). It might interfere with the saliva secretion and composition(15). There is some question regarding the theoretical basis of salivary ferning, in that ferning is also discovered in male saliva(24) and can be found throughout the female cycle(36).

In conclusion, these findings suggest that the saliva ferning test may be unreliable for predicting the ovulatory period in clomiphene citrate-stimulated cycles and its use should, therefore be discouraged. Further study of salivary ferning test in different purposes may have benefit in special conditions.

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การทดสอบการตกผลึกกิ่งใบเฟิร์นในน้ำลายและการตกไข่ในรอบระดูที่กระตุ้นด้วยยาโครมิฟินซิเตรท

สุเมธ พัฒนาสุทธินันท์, วิสันต์ เพชรพงศ์, สมชาย สุวจนกรณ์

วัตถุประสงค์: เพื่อกำหนดวันไข่โดยการทดสอบตกผลึกรูปใบเฟิร์นในน้ำลาย ในรอบระดูที่กระตุ้นด้วยยาโครมิฟินซิเตรท

รูปแบบการวิจัย: การวิจัยเชิงพรรณนา

สถานที่: ภาควิชาสูติศาสตร์-นรีเวชวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

กลุ่มตัวอย่าง: ผู้ที่มีการยืมตัวอย่างจาก 75 รายซึ่งมีรอบสม่ำเสมอ

วัสดุและวิธีการ: ผู้ที่มีการยืมตัวอย่างโดยยืมจากนกฮูก ซิเตรท ขนาด 100 มิลลิกรัมต่อวัน เป็นเวลา 5 วัน แล้วเก็บตัวอย่างน้ำลายทุกวันจนกระทั่งเกิดไข่ แล้วทำการตรวจคลื่นเสียงความถี่สูงทุกวันเพื่อกำหนดวันไข่ ทำการทดสอบตกผลึกกิ่งใบเฟิร์นในน้ำลายทุกวันที่จะเกิดไข่ ทุกอย่างจะเกิดไข่ 7 วันหลังไข่ แล้วเก็บตัวอย่างน้ำลายทุกวันจนกระทั่งเกิดไข่ แล้วทำการทดสอบตกผลึกกิ่งใบเฟิร์นในน้ำลายทุกวัน หลังจากเกิดไข่ วันที่มีการตกผลึกกิ่งใบเฟิร์นสูงสุด วันไข่จากกิ่งใบเฟิร์นสูงสุด วันไข่ จากกิ่งใบเฟิร์นสูงสุด วันไข่ จากกิ่งใบเฟิร์นสูงสุด วันไข่ จากกิ่งใบเฟิร์นสูงสุด

ผลการศึกษา: อายุและความยาวรอบระดูของผู้ป่วยแสดงเป็นค่าเฉลี่ยเกิน 32.9 ± 3.7 ปี และ 28.4 ± 1.3 วัน พบว่าวันที่มีการตกผลึกกิ่งใบเฟิร์นสูงสุดตรงกับวันไข่จากการตรวจคลื่นเสียงความถี่สูง 7.1% เห็นได้ว่ามีประสิทธิภาพของเฉพาะการทดสอบตกผลึกกิ่งใบเฟิร์นสูงสุด 2 วัน คือมีค่าเรียกเห็นสูงสุดที่ตรงกับวันไข่และวันไข่เกิดไข่ ไม่พบความสัมพันธ์ระหว่างวันที่มีการตกผลึกกิ่งใบเฟิร์นสูงสุดกับวันไข่ (r = 0.102, p > 0.05)

สรุป: รอบระดูที่กระตุ้นด้วยยาโครมิฟินซิเตรท ในการทดสอบการตกผลึกกิ่งใบเฟิร์นในน้ำลายสูงสุดจะไม่สัมพันธ์กับการตกไข่.