Ovulation Detection in Saliva, Is It Possible?

A. Salmassi    A.G. Schmutzler    F. Püngel    M. Schubert    I. Alkatout    L. Mettler

Department of Obstetrics and Gynecology, Unit of Reproductive Medicine, University Hospitals Schleswig-Holstein, Campus Kiel, Kiel, Germany

Key Words
Ovulation detection in saliva · Mini-microscope

Abstract

Background/Aims: The new mini-microscope Geratherm® ovu control was evaluated for its recognition of saliva ferning in a collective of 47 patients taking part in an artificial reproductive technology program on the day of follicular puncture. Methods: The ferning phenomenon was evaluated by patients and laboratory staff according to the criteria: no ferning, slight ferning and good ferning. Results: Geratherm® ovu control showed a specificity of 78% and a sensitivity of 80% in relation to rising E₂ levels under follicle-stimulating hormone/human chorionic gonadotrophin. A comparison of the evaluations of the saliva test carried out by patients and by laboratory staff resulted in a high and substantial agreement of 89.4% (κ). Conclusion: Evaluations performed by ovu control were similar to those achieved with a highly sophisticated inverted microscope.

Introduction and Aim of the Study

In 1945, Papanicolaou observed microscopic crystal formations in cervical mucus and described this pattern as ferning. Subsequent researchers have studied the ferning phenomenon of other body fluids, including saliva [1–4]. The crystallization of saliva showed the ferning to be virtually identical in appearance to the arborization effect of cervical fluid. The saliva test has been used to ascertain a woman’s fertile period with a success rate of >90% [5]. In 1972 our group in Kiel verified the ferning phenomenon in cervical mucus according to Insler et al. [6]. Further studies examined the use-effectiveness of salivary ferning as a diagnostic testing aid in natural family planning [7, 8].

It was the aim of the present study to show the correlation of saliva ferning in the preovulatory period, on the day of follicular puncture (FP), to the E₂ levels, egg collection and fertilization results in patients taking part in an in vitro fertilization (IVF), intracytoplasmatic sperm injection (ICSI) and embryo transfer (ET) program. These patients were stimulated with the recombinant follicle-stimulating hormone (rFSH, Gonal F; Merck Serono, Munich, Germany) and ovulation was induced with human chorionic gonadotrophin (hCG; Merck). The phenomenon of saliva ferning was assessed by patients and laboratory staff with the mini-microscope Geratherm® ovu control (ovu control). To test the performance of ovu control, additionally prepared saliva slides were evaluated under a highly sophisticated inverted microscope.

This easy tracking of saliva ferning in artificial reproductive technology (ART), dependent on the estradiol rise and LH surge, can easily be extrapolated to natural cycles pinpointing the time of ovulation without medical assistance to plan intercourse within the home surroundings.
Fig. 1. Disposable mini-microscope Geratherm® ovu control. A drop of saliva is deposited on the lens, air dried and evaluated 15 min later by holding the microscope in front of the eye to judge the ferning structures: negative = 0, slight ferning = 1+, and good ferning = 2+ (fig. 1).

Material and Methods

General Remarks and Institutional Review Board Approval

47 females, aged 18–42 years, participated in this salivary ferning study during their FSH/hCG stimulation treatment at the Unit of Reproductive Medicine, Department of Obstetrics and Gynecology, Campus Kiel, Germany, in 2012. All patients signed the consent form in accordance with the requirements of the ethics committee of the University of Kiel. The study was approved by the institutional review board.

Patients

Saliva and serum were collected on the day of FP from 47 infertile patients. The patients were aged 20–42 years (median 33) and the size of the lead follicle on the day of FP measured 19–24 mm.

IVF Stimulation and Ferning Assessment in Saliva

Patients undergoing IVF/ICSI were stimulated with rFSH and downregulated with the gonadotrophin-releasing hormone agonist Synarel (GnRH; Pharmacia, Erlangen, Germany) in the long protocol. Follicular development was measured by real-time ultrasound scans and serum E2 levels from day 6 of stimulation until the day of hCG application. Before the leading follicle measured >17 mm in diameter and the 17β-E2 level was adequately increased but still <3,000 pg/ml in serum, 6,500 IU of hCG were administered subcutaneously. The number of follicles was determined on the day of ovulation induction by hCG. Progesterone (Pr) levels were measured parallel to E2 and LH. Early increased levels of Pr and LH indicated an earlier FP timing with hCG than was routinely performed, 36 h prior to the morning FP between 8 and 11 a.m. Follicles were aspirated 36 h after administration of hCG.

After ET the patients were treated with Pr vaginally (Utrogest, 600 mg daily; Dr. Kade/Besins, Berlin, Germany) for luteal support until confirmation of pregnancy by β-hCG determination.

Collection of Serum and Saliva Samples

Blood samples were taken from all patients, centrifuged for 10 min at 350 g and 5°C, shock-frozen and kept at –80°C.

Geratherm® Ovu Control

Saliva samples were evaluated on the day of FP on the ocular of ovu control and on two slides under an inverted microscope. The evaluation of the ferning pattern with the mini-microscope ovu control was performed by the patients and the laboratory staff according to three criteria: negative = 0, slight ferning = 1+, and good ferning = 2+ (fig. 1).

Evaluation of Ferning by Laboratory Staff

The evaluation of the ferning on the slides was performed only by the laboratory staff. The patients were asked to first perform the ovu control test and then put a drop of saliva on two prepared slides, air dry them and give them to the laboratory staff on the next day for evaluation of ferning under the inverted Zeiss microscope (magnification 100–200×). Two laboratory staff judged the ferning according to the same criteria as described above and then determined the cumulative mean judgement in the laboratory.

Biochemical Analyses

Pr, LH and E2 Assay in Serum

Pr, LH and E2 levels were measured by a solid-phase, competitive chemiluminescent enzyme immunoassay with the Immulite 2000 Auto System (DPC-Biermann, Siemens, Germany) within the range of 0–2,000 pg/ml for E2 (analytical sensitivity 15 pg/ml), 0.2–40 ng/ml for Pr (analytical sensitivity 0.2 ng/ml) and 0–200 mIU/ml for LH (analytical sensitivity 0.05 mIU/ml).

Statistical Evaluation

We compared the E2 level on the day of FP to the patient’s evaluation with ovu control and determined cut-off levels for sensitivity and specificity. In a first step, the patient’s observation of ferning was compared to the laboratory staff’s observation with ovu control. In a second step, the laboratory staff’s evaluation of saliva smears on slides using the inverted microscope was compared to the patient’s ovu control evaluation. In a third step, the laboratory staff compared their observations with ovu control to their observations with the slides under the inverted Zeiss microscope.

Pearson’s correlation coefficient (r) was applied to investigate the correlation between saliva assessment by patients with ovu control and their observations with the slides under the inverted Zeiss microscope.

We used the κ values to measure the interobserver agreement [9–11]. A κ of 1 indicates perfect agreement, whereas a κ of 0 indicates agreement equivalent to chance. A limitation of κ is that it is affected by the prevalence of the finding under observation [11, 12]. κ agreement is interpreted as follows: <0 = less than chance agreement, 0.01–0.20 = slight agreement, 0.21–0.40 = fair agreement, 0.41–0.60 = moderate agreement, 0.61–0.80 = substantial agreement, and 0.81–0.99 = almost perfect agreement.
To evaluate the E_2 cut-off levels for the observation of positive and negative saliva ferning by ovu control, we used ROC-AUC (receiver operator characteristic curves-areas under curve) with minimized false positive and false negative results.

**Results**

**Patient Parameters**

Table 1 gives an overview of the mean levels and standard deviations (±SD) of age, BMI, total injected dose of rFSH up to the day of hCG injection, E_2, Pr values, number of retrieved oocytes on the day of FP and number of fertilized oocytes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean level ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of women</td>
<td>35.06±4.4</td>
</tr>
<tr>
<td>BMI of women</td>
<td>24.26±4.7</td>
</tr>
<tr>
<td>Stimulation days</td>
<td>12.2±2.1</td>
</tr>
<tr>
<td>Total dose, IU</td>
<td>3,106.6±1453.2</td>
</tr>
<tr>
<td>Estradiol, pg/ml</td>
<td>947.75±511.7</td>
</tr>
<tr>
<td>Progesterone, ng/ml</td>
<td>7.15±3.12</td>
</tr>
<tr>
<td>Number of follicles</td>
<td>16.6±8.7</td>
</tr>
<tr>
<td>Number of oocytes</td>
<td>10.2±5.4</td>
</tr>
<tr>
<td>Number of fertilized oocytes</td>
<td>7.8±3.1</td>
</tr>
</tbody>
</table>

**Evaluation of Hormone Levels**

Table 2 shows the evaluation of Pr, LH and E_2 levels in serum of 47 patients throughout the ovarian stimulation cycle and up to the day of FP. A gradual increase of Pr and E_2 from day 6 through to day 10, reaching a peak on the day of hCG injection, is evident. On the day of oocyte retrieval, the E_2 levels had already dropped significantly. The differences within these groups, as analyzed by the Friedman test, were significant for Pr (p = 0.027) and E_2 (p = 0.007). There is no variation in LH levels.

<table>
<thead>
<tr>
<th>Stimulation days</th>
<th>Pr ± SD, ng/ml</th>
<th>LH ± SD, IU/ml</th>
<th>E_2 ± SD, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.38±0.16</td>
<td>1.4±1.25</td>
<td>157.1±116.18</td>
</tr>
<tr>
<td>8</td>
<td>0.47±0.18</td>
<td>1.31±1.03</td>
<td>657.22±426.14</td>
</tr>
<tr>
<td>10</td>
<td>0.55±0.23</td>
<td>1.3±1.16</td>
<td>1,214.3±624.56</td>
</tr>
<tr>
<td>Day of hCG injection</td>
<td>1.12±0.78</td>
<td>1.68±1.18</td>
<td>2,568.6±119.6</td>
</tr>
<tr>
<td>Day of FP</td>
<td>7.15±3.12</td>
<td>1.1±0.3</td>
<td>947.7±511.7</td>
</tr>
<tr>
<td>p</td>
<td>0.027</td>
<td>NS</td>
<td>0.007</td>
</tr>
</tbody>
</table>

**Analysis of Observation and Assessment of Saliva Ferning**

Comparison of Observation of Saliva Ferning with Ovu Control by Patients and Evaluation with the Microscope by Laboratory Staff

Table 3 shows the comparison of the assessment of saliva ferning between patients with ovu control and laboratory staff for testing with ovu control (fig. 2).

<table>
<thead>
<tr>
<th>Observations of laboratory staff (ovu control)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observations of patients (ovu control)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>22</td>
</tr>
<tr>
<td>Positive 1+</td>
<td>2</td>
</tr>
<tr>
<td>Positive 2+</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
</tr>
</tbody>
</table>

Comparison of Observation of Saliva Ferning with Ovu Control by Patients and Evaluation with the Microscope by Laboratory Staff

Table 4 shows the comparison of the assessment of saliva ferning between patients with ovu control and laboratory staff with a microscope (magnification 100–200×). The saliva ferning was classified as: negative, positive 1+ or positive 2+. There was a 59% consensus (28/47) between the two observers. We found a positive and significant correlation between the two observers (r = 0.66, p < 0.001, χ²) between the two observers. The κ level amounted to 0.644 which indicates a substantial agreement.

**Table 1. Mean level ± SD of age, BMI, total injected dose of rFSH up to the day of hCG injection, E_2, Pr, number of retrieved oocytes on the day of FP and number of fertilized oocytes**

**Table 2. Mean level ± SD of Pr, LH and E_2 in serum of patients (n = 47) during the stimulation phase and on the day of FP; the differences within these cycle phases as analyzed by the Friedman test**

**Table 3. Comparison of the assessment of saliva ferning with ovu control between patients and laboratory staff**

**Table 4. Comparison of the assessment of saliva ferning between patients with ovu control and laboratory staff with a microscope (magnification 100–200×)**

Ovulation Detection in Saliva, Is It Possible!
Combining positive 1+, positive 2+ and all matching observations of saliva ferning, we found an agreement of 82.9% (39/47) between observations by patients with ovu control and by laboratory staff with the sophisticated microscope.

A positive and significant correlation (r = 0.69, p < 0.001) between the observers was detected. The κ level amounted to 0.67 which indicates a substantial agreement.

Comparison of the Laboratory Staff’s Observation of Saliva Ferning with Ovu Control and with the Microscope

In this analysis we compared the assessment of saliva ferning with ovu control to the slide evaluation under the inverted microscope. We found a 65% consensus (32/47) between the two assessment methods (table 5).

There is a positive and significant correlation according to Pearson (r = 0.72, p < 0.001) between the two microscopes. The κ level amounted to 0.55 which indicates a moderate agreement.

However, combining positive 1+, positive 2+ and all matching observations of saliva ferning, an agreement of 85.1% (40/47) results if experts (laboratory staff) perform the evaluation.

A positive and significant correlation (r = 0.69, p < 0.001) was found between the two observers. The κ level amounted to 0.703 which indicates a substantial agreement.

Serum E₂ Values Compared to Saliva Positivity with Ferning Phenomenon

The assessment of serum E₂ values on the day of FP compared to the ovu control saliva ferning measurements are best evaluated by the E₂ cut-off levels in serum. The typical ROC-AUC for E₂ indicating the saliva ferning in ovu control reveals a high sensitivity and specificity (fig. 3).

As seen in figure 3 and table 6, according to the ROC-AUC we found a sensitivity of 80% and a specificity of 78% at serum E₂ cut-off levels of 793 pg/ml.

At E₂ levels >793 pg/ml, 75% positive and 25% negative observations for ferning with ovu control were detected. At E₂ levels <793 pg/ml, 37% positive and 63% negative observations were identified (p = 0.03).
Discussion

The results of this study can be compared to a number of previous studies correlating positive ferning in cervical mucus and saliva to the fertile period of the female (3- to 4-day period) [2, 6, 13, 14]. However, no specificity or sensitivity was reported. This study, however, with induced ovulation and assessment of ferning on the day of FP in an ART program shows the direct correlation to an average of 10 aspirated mature oocytes. While predicting and detecting ovulation is of great importance for those who wish to conceive or avoid a pregnancy, it should be stated that all semiquantitative tests in urine, cervical mucus or saliva have their limitations. ovu control is a semiquantitative or qualitative test and gave interesting results [15].

This study, performed in a group of females stimulated with rFSH in an IVF/ET program at the Unit of Reproductive Medicine, University of Kiel, evaluated Geratherm® ovu control, which detects salivary ferning patterns, by comparing its performance with that of evaluation through a highly sophisticated inverted microscope.

It was interesting to find a high agreement between well-trained laboratory staff and patients in judging the ferning test as only positive or negative. We found a positive and significant correlation according to Pearson ($r = 0.69, p < 0.001, \chi^2$) between the two observers. The $k$ level amounted to 0.664 which indicates a substantial agreement.

Comparing the $E_2$ values to positive saliva ferning on the day of FP gave a sensitivity of 80% and a specificity of 78%, at a cut-off level of 793 pg/ml. This compares well with other qualitative estimations from urine, cervical mucus or saliva for the fertile days of the female, in other words for the approximation to ovulation during the menstrual cycle. Using a comparable mini-microscope to ovu-control, Cleofe Medina found a sensitivity of 53% and a specificity of 73% for saliva ferning in relation to the LH surge [16–18].

The clinical benefits of Geratherm® ovu control in saliva are that (1) the saliva ferning phenomenon is an additional clinical method for detecting ovulation and avoids daily drawing of blood as is necessary in serum testing of estradiol or LH, and (2) compared to other substances measured during the menstrual cycle, such as serum sclerostin [19], no influence of sex hormones could be detected.

Conclusions

The application of Geratherm® ovu control for observation of salivary ferning patterns in patients undergoing rFSH treatment showed a specificity of 78% and a sensitivity of 80% in relation to the rising $E_2$ levels under FSH/hCG stimulation on the day of FP. We found an extremely high and substantial agreement of 89.4% between the observations of patients and laboratory staff for saliva testing with ovu control.
References