

## Daily Saliva and Vaginal Mucins Co-vary in Protein-to-Carbohydrate Ratio

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### Abstract

The currently reported study employed the curiously named (but simple) quantitative, nondestructive technique of Multiple Attenuated Internal Reflection InfraRed (MAIR-IR) spectroscopy to assess daily variations of both saliva and vaginal secretions from a panel of consenting volunteers. The project goal was to determine whether the protein-to-carbohydrate (starch) ratio, determined by MAIR-IR analysis of salivary and vaginal secretions is an objective measure of the co-variation of mucinous secretions with daily menstrual cycle. Overall, the spectral data were found to provide valid signatures of vaginal mucus chemical changes that also correlated with cyclic periods of presumed fertility, such periods exhibiting repeat times of 14-16 days and 27-29 days in the thoroughly analyzed records. At mid-cycle, associated with ovulatory events, the protein-to-carbohydrate ratio of vaginal mucin maximizes. Higher carbohydrate ratios are associated with stiffer cervical mucus plug formation, which usually prevents infection, spontaneous abortion, and pre-term birth, but may also be responsible for infertility. Mucin relaxation compounds, such as the FDA-approved oral rinse, delmopinol, may induce fertility in such cases.

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**Running Title:** Mucin Variations Reflect Fertility

**Keywords:** glycoprotein, infrared analysis, periodicity, polysaccharide, saliva, secretions, spectroscopy ratios

**Received:** Aug 11, 2018

**Accepted:** Sep 20, 2018

**Published:** Sep 20, 2018

**Editor:** Roman Kireev, PhD, Senior Researcher, Spain.

## Introduction

Self-reports of menstrual cycle symptoms have long been criticized, calling for more objective measures that could correlate better with physiological events.<sup>1</sup> It is obviously important that, if cervical mucus specimens are to be the criteria objectively evaluated, there must be a proof of their stability while introduced to the analytical methods.<sup>2</sup> In the mucin glycoproteins, poly-anionic groups (mainly sialic acid) are carried on oligosaccharides (short starch segments) of about 9-10 monosaccharide lengths, but the chemical and serological differences in oligosaccharide structure were not previously reported to have any biological function.<sup>2</sup> This generally pessimistic view is challenged by a plethora of prior studies of salivary mucins, very similar to cervical glycoproteins, that do illustrate structural and functional changes (some of which co-vary with menstrual cycle days) with modified compositions.<sup>3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13</sup> Although carefully controlled saliva sampling regimens do demonstrate this cyclic variation, normal oral activities, eating, and drinking can obscure the same correlation that may be demonstrated more reliably by vaginal mucin smears.

Such cyclical changes may serve as indicators of both shorter and longer term fluctuations in states of human fertility, health, and disease.

## Experimental Procedure

This report describes investigative research into cyclical changes that occur in protein/starch (P/S) ratios of intraoral glyco-proteins and vaginal mucins among humans, as characterized by Multiple Attenuated Internal Reflection InfraRed (MAIR-IR<sup>14</sup>) spectroscopy. In addition to observing P/S relationships over several menstrual cycles, the investigation repeated examination of ratios within 24-hour time-spans to determine whether the variance in sampling times altered the sample results and to identify the amount of change that normally occurs in the P/S ratio on a daily basis.

To obtain a broad sample group for the study, 15 mid-life female volunteers were selected (by acknowledged collaborators) based on differences in age, medical history and medications used, diet, health habits, contraceptive techniques used, and frequency of sexual activity. Each volunteer, identified only by number and otherwise unknown to the investigators,

provided intra-vaginal and/or intra-oral samples on a regular basis and recorded pertinent data when each sample was taken. Each sample was then analyzed using internal reflection (infrared) spectrometers and the P/S ratios for the samples were plotted on graphs ranging from one to six months. This study period covers oral and vaginal specimens during the period from January 1978 through January 1980. Additional saliva specimens were obtained from one new volunteer in 2008-2009 through one menstrual cycle in order to compare the quality of infrared spectra obtained nearly 30 years earlier with spectra obtained with modern equipment.

### *Specific objectives were*

- a) Collect samples of mucous secretions from human volunteers at regular intervals.
- b) Using MAIR-IR spectroscopy, determine the relative abundance of protein and carbohydrate moieties, to obtain a chronological record of the variability of the chemistry of these secretions.
- c) Correlate these mucin chemistry data with other variables, such as onset of menstruation, diet, dental plaque formation, medication and physical trauma.

Prior to participation in the study, each volunteer was advised of the nature of the project, its scope, the sampling techniques to be used and the confidentiality of the data to be provided, in accordance with directives from the approving institutional Human Subjects Committee. It was also determined that the sterile cotton swabs for taking the samples would not pose any significant health hazard to the project participants. All participants were required to sign waiver statements and consent forms and were assigned participant numbers to preserve the confidentiality of all collected data.

## Materials and Methods

Each of the participants was given a supply of sterile, culture collection swabs. Each mucin sample was taken by inserting a just-opened sterile swab directly into the vagina or the mouth. After removal from the body cavity, the swab was returned to its original sterile tube and a sample label was prepared. The label identified the participant's number, as well as the date and time at which the sample was taken.

In addition to labeling of samples, volunteer participants assisted by filling out a brief medical questionnaire, which was returned to the project investigator after all sampling was completed; and an event history form, which outlined medication intake, amount of sexual activity, and other variables that might alter a specimen's analytical result.

After collected samples were received from the volunteer participants, each sample swab was used to apply a thin-layer smear to the surface of a germanium internal reflection prism, compensated to exhibit a 96% transmission at  $2000\text{ cm}^{-1}$ . Each sample was analyzed using Perkin-Elmer 710B and Perkin-Elmer 700 infrared (IR) spectrometers with internal reflection mirror assemblies. Information from each sample label was also filled in on the corresponding spectrometer graph and the graph was included in a note-book file maintained for each participant. P/S ratios were determined from the IR spectra of the samples and were plotted as points on graphs representing from one to six months of sampling. Numerous spectral analytical procedures were then applied to seek periodicities.

To measure each specimen's P/S ratio, the printout of the sample spectrum was compared to the baseline spectrum of the prism, as a percentage IR transmission value, at both the starch and protein peaks. An example of a sample spectrum is given in Figure 1. The protein/baseline % transmission was then divided by or subtracted from the starch/baseline percent transmission, result in P/S or P-S ratios. Two independent assays of each specimen were recorded and the average of the replicate ratios was then plotted for each day.

Calibration procedures were carefully applied. Throughout this investigation, several control experiments were set up to help identify the parameters of the study. Each experiment was intended to control one aspect of the study. Baseline measurements also were taken on a daily basis by analyzing the clean germanium prisms in the spectrometers prior to any sample testing.

## Results

Over 2000 individual spectral records were obtained for mucin samples from approximately 20

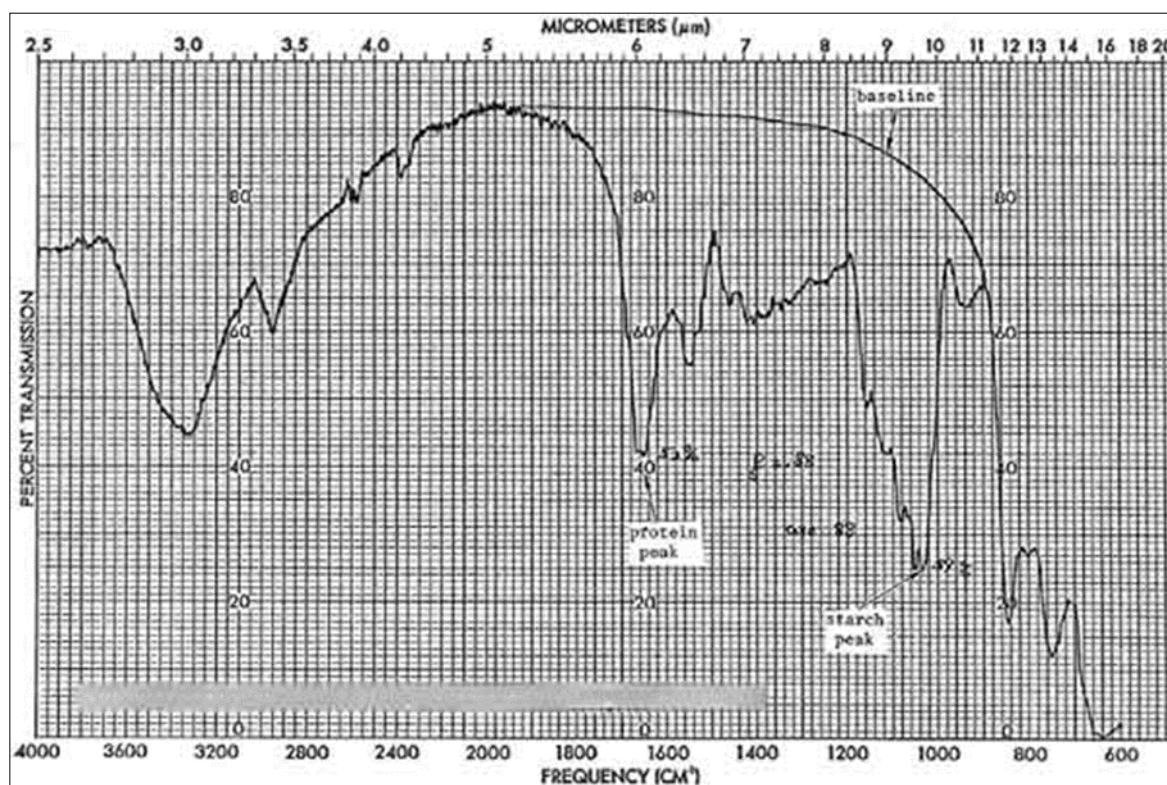


Figure 1 - A typical InfraRed (IR) Spectrometer plot for energy absorption by a mucin specimen, showing dominant absorption bands for both protein and starch (carbohydrate).

human subjects, with saliva samples collected more regularly in recent years. The large majority of these samples reflected the daily variation of vaginal mucus glycoprotein composition. Many of the early saliva studies were terminated when it was learned that the generally lower-viscosity intraoral fluids were retained within the cotton tips of the sampling swabs and could not be reproducibly applied to the analytical prisms. Figure 2, however, is a plot of data from the saliva samples characterized in 2008-2009 (one volunteer), that does illustrate the sought correlation with menstrual cycle day. In parallel studies at the University of California, San Francisco, and Malmo University, Sweden, it was found that similar cyclic variation of the surface properties (due to adsorbed salivary mucoid components) of human teeth could be correlated with the menstrual cycle of female volunteers. Male volunteers did not show this variation in tooth surface chemistry when repetitively tested.<sup>8</sup>

Overall, the spectral data were found to provide valid signatures of vaginal mucus chemical changes that also correlated with cyclic periods of presumed fertility, such periods exhibiting repeat times of 14-16 days and 27-29 days, in the thoroughly analyzed records. Considerable computer-aided spectral record analysis was required to verify these findings, which still require independent confirmation from other laboratories.

*Variation with Age, Contraceptive Technique and Sexual Activity*

Female volunteers accepted into the study ranged in age from 22 to 43 years, with most being in the age group of 24-26 years. The volunteers reported a range in frequency of sexual intercourse from none to 4 times weekly throughout the study period. Contraceptive techniques practiced ranged from none through the use of condoms, and regular as well as irregular use of hormonal drugs (the "pill"), use of diaphragms, and use

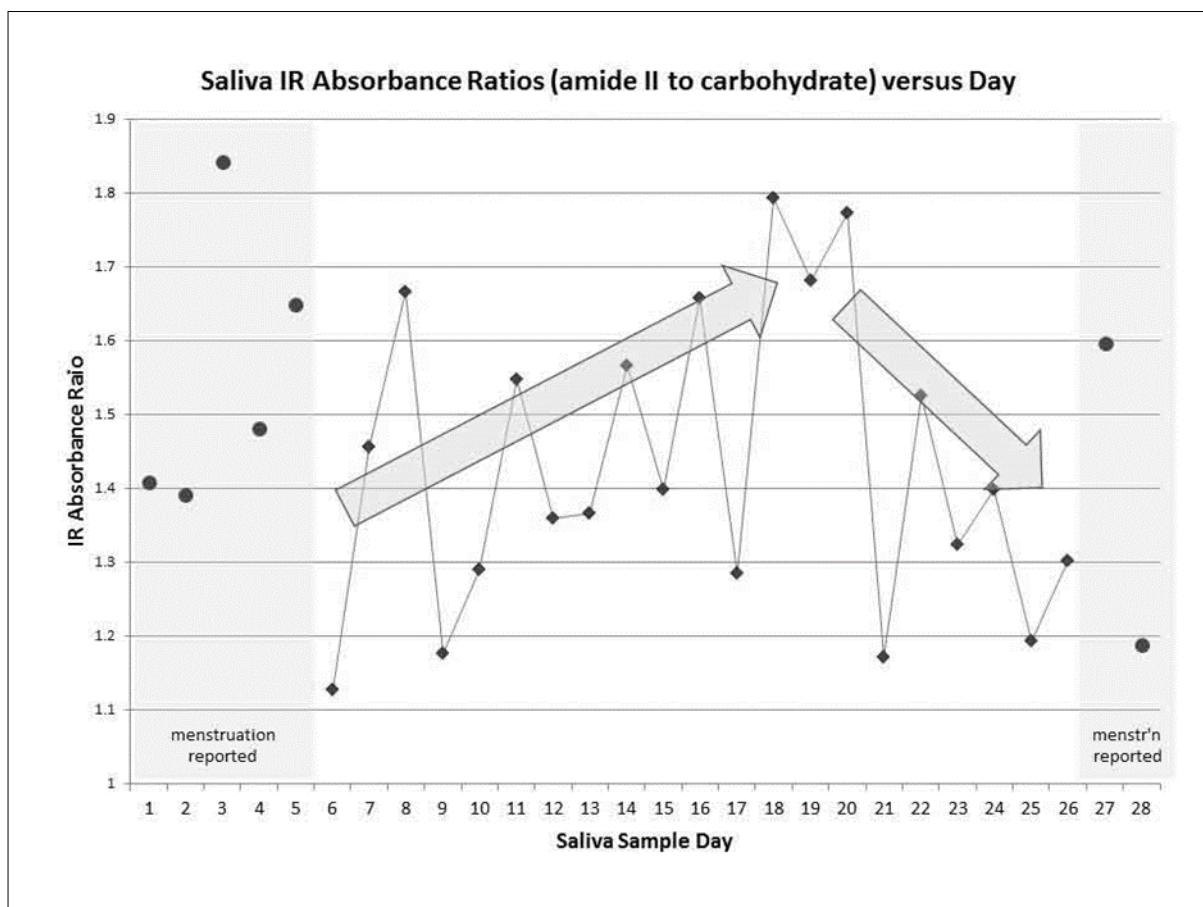


Figure 2 - The daily IR variation of protein-to-starch ratio for saliva of a fertile woman, with care taken to avoid extraneous oral compounds (e.g. food, drink, medicine). Note the clear correlation with the mid-point of the menstrual cycle.

of intrauterine devices. Tampon as well as absorptive pad users provided samples daily, even throughout the menstrual period.

With the exception of samples obviously mixed with menstrual blood or residual semen, the vaginal mucus specimens from each volunteer showed repeating twice-monthly shifts in the relative abundance of protein and starch components. The baseline above and below which these shifts were noted was clearly different for different subjects, with the average protein

content being relatively highest in the oldest subject.

Figure 3 provides a group of 5 spectral records for one subject, typifying the variation seen in mid-cycle. Note that the considerable shift in apparent protein-to-starch content of the vaginal mucus at about Day 14 correlates well with the time of ovulation, and that secondary physiochemical changes in the mucus itself -- notably its viscosity and "stringiness" -- also reflect these changes.<sup>15, 16</sup>

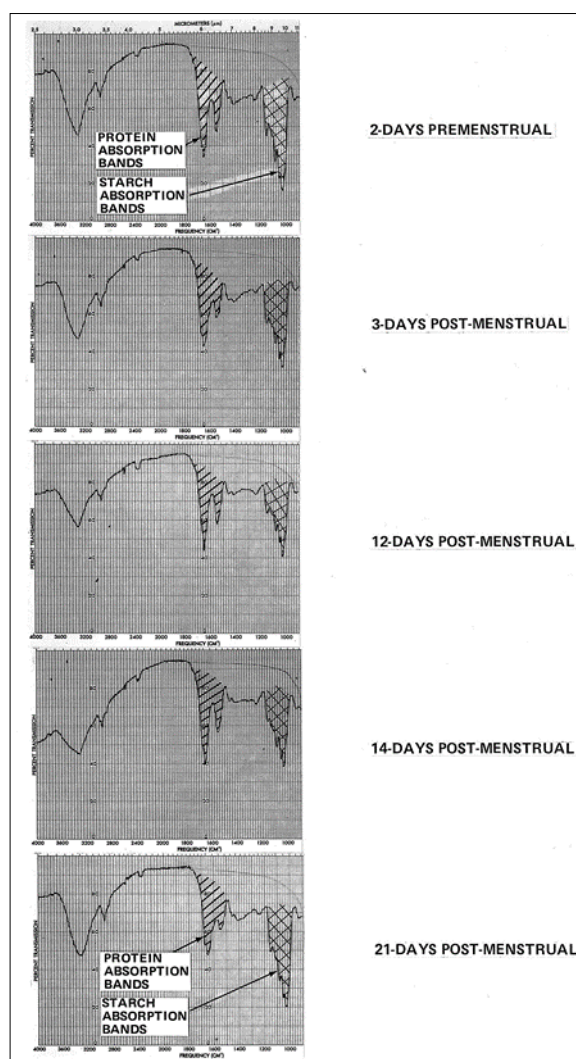


Figure 3 - A comparative illustration of the actual IR spectral variations in daily specimens from a vaginal mucin donor, arranged from top to bottom in relation to reported menstrual cycle day (consider Day 1 as the first day of that person's menstrual flow for that month).

*Reliability and Range of the Data*

Figure 4 provides two spectral comparisons of the identical vaginal mucus specimens as analyzed using the two different spectrophotometers used during the 1978-1980 study period. As illustrated, there were occasional shifts in the relative proportions of the diagnostic protein and starch bands as analyzed by the different instruments. Thus, the decision was made to limit the analyses of any given subject's specimens to a single instrument, and that recommendation is also cited here.

It was also of concern, recognizing the general availability of only one specimen per subject per day, to determine the single day variability of vaginal mucus contents so that only variations beyond that amount would be considered significant. Thus, one volunteer was asked to provide repeated specimens within a single 24-hour timeframe. Sixteen samples taken at roughly hourly intervals over a total period of 21 hours revealed a variability in protein-to-starch ratio as follows: 0.87, 0.87, 0.76, 0.76, 0.81, 0.82, 0.88, 0.86, 0.75, 0.79, 0.78, 0.81, 0.81, 0.81, 0.86, 0.90. In a separate

experiment, using different germanium internal reflection prisms for individual samples taken over an 8-hour period, the protein-to-starch ratio varied only from 0.88 to 0.94. Working with the same specimen swab, which usually carried more than enough mucus for multiple analyses, variations in amounts of mucus applied to analytical prisms and in the degree of distribution of the specimen over the prism face did not significantly affect the protein-to-starch ratios observed, even though the overall spectral contrast did vary directly with amount of sample applied and its uniformity of distribution over the test plate.

Accepting the further limitation that all samples could not be provided or analyzed immediately after collection, it was necessary to establish that time delays between sampling and analysis did not seriously skew the data records. Samples were, therefore, analyzed immediately as taken from the body cavity, and then at repeated intervals (using the same swab stored in its sterile plastic container) for 7 days. The protein-to-starch ratios varied from 0.88 to 0.95 in a typical case.

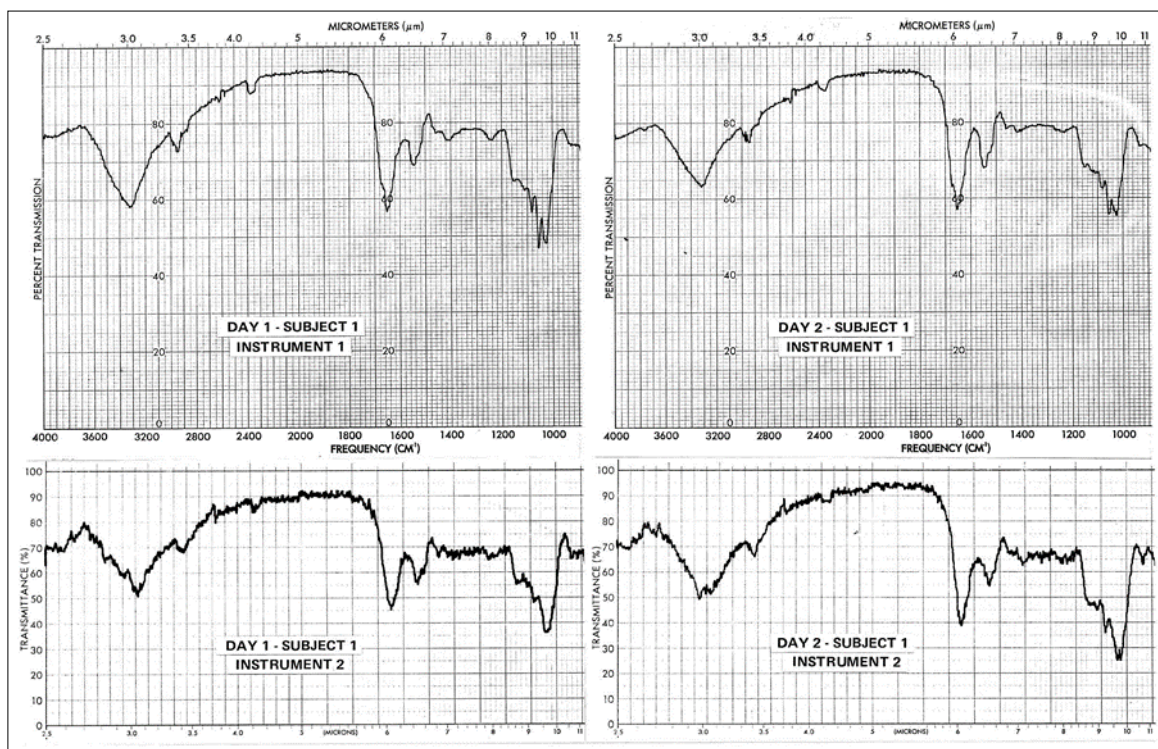


Figure 4 - Two spectral comparisons of the same vaginal mucin samples analyzed by the reported MAIR-IR technique, using two different laboratory instruments, illustrating the general reproducibility of the findings.

It was also of interest to know the analytical variation that might be attributed to sample manipulation, instrumentation drifts, and changes in sample character once applied to a given analytical prism. To study these variables, single specimens were applied to different analytical prisms, and -- within the same day -- analyzed, removed from the instrument, stored while the instrument was used for other purposes, and remounted for duplicate analyses. Typical duplicate analyses (protein-to-starch ratio) obtained for specimens taken many days apart are as follows: 1.41 vs 1.48; 0.94 vs 0.96; 1.07 vs 1.04; 0.89 vs 0.86; 0.76 vs 0.77; 0.65 vs 0.65. The conclusion must be that that IR monitoring of mucin specimens is a reliable method.

Of all possible sources of error or variation in the analytical scheme, none was judged large enough to invalidate the cyclic changes noted during the study to correlate with the apparent fertile periods of the volunteers. For example, for one subject providing specimens daily for a seven month period, the protein-to-starch ratios varied from as low as 0.65 to as high as 1.69, with the entire range covered within even a single month. For another volunteer, monthly variations in the protein-to-starch ratio were as follows: January - 0.95 - 1.50; February - 0.94 - 1.36; March - 0.94 - 1.3; April - 0.70 - 1.20; May - 0.79 - 1.15; June - 0.88 - 0.97; July - 0.76 - 0.91. Illustrating the differing baselines around which these variations occurred for different women, one subject had a protein-to-starch ratio variation of 1.2 - 3.8 over 3 months, another of 0.9 - 2.0 over 2 months, and yet another 0.8 - 2.8 over 6 months.

Figure 5 provides spectral records of a significant aberration in these values as a reflection of physical trauma, brief hospitalization, and medication experienced by one volunteer. Using only desk-top methods for data reduction, Figures 6 and 7 show crude curve-fitting attempts for data (protein - starch absorption values, as read directly from the spectral traces) including a clear anomalous period due to subject trauma and medication. As revealed in Figures 6 and 7, this volunteer's trauma had an extreme, but reversible, impact on the chemical nature of her vaginal mucus. The inadvertent demonstration of the reflection of physical and medicinal changes of health (and, possibly, emotional) status in the chemistry of human

vaginal mucus suggests that regular monitoring of such mucus specimens may become a significant aid in medical diagnosis of ills only indirectly related to problems of fertility and sterility.

## Discussion

It has long been suspected that salivary mucin properties, such as viscosity,<sup>17</sup> reflect the time of ovulation, but this (like the relative clarity or opacity of the actual cervical mucus) generally has had no prognostic significance. Still, it has long been considered probable that quantitative or qualitative alterations in mucin glycoproteins are under hormonal control,<sup>18</sup> and also probably involved in sperm capacitation.<sup>19, 20, 21</sup>

The study reported here demonstrated that the protein-to-carbohydrate ratio of vaginal mucin maximizes at mid-cycle, associated with ovulatory events. This event is correlated with known physical changes allowing easier sperm passage, apparently the result of carbohydrate chain changes via glycosyl oxidases.

The oligosaccharide (carbohydrate, starch) variations in proportion to their host protein contents were significant and reproducible with cycle day in this study. The direct, nondestructive, observation of daily variable carbohydrate (starch) of 60%-80% weight ratio to the parent mucin protein (20%) core does not necessarily relate to the excess sugar often detected in saliva at the ovulatory time<sup>22, 23</sup> but this may occur via oligosaccharide side-chain shortening by activated glycoside oxidases. The oligosaccharide comprise the major sugars N-acetylglucosamine, N-acetylgalactosamine, galactose, sialic acid, and fucose.<sup>24</sup>

As these mucins imbibe water to become gel-like mucus,<sup>25</sup> they have large expansions that fill space and can block the cervical opening, preventing sperm transport at pre- and post-ovulatory times.<sup>26</sup> Thus, variable carbohydrate chain lengths of mucins stratify the probabilities for both initial fertility and for later preterm birth<sup>27</sup> or spontaneous abortions.<sup>27, 28</sup> Defined patterns of glycosylation are also associated with mucins in the respiratory tract.<sup>29,30</sup> The successful modulation of stiffness of lung sputum by an FDA-approved saliva - mucin - coagulating dental plaque-removing rinse<sup>31</sup> suggests that this therapy may

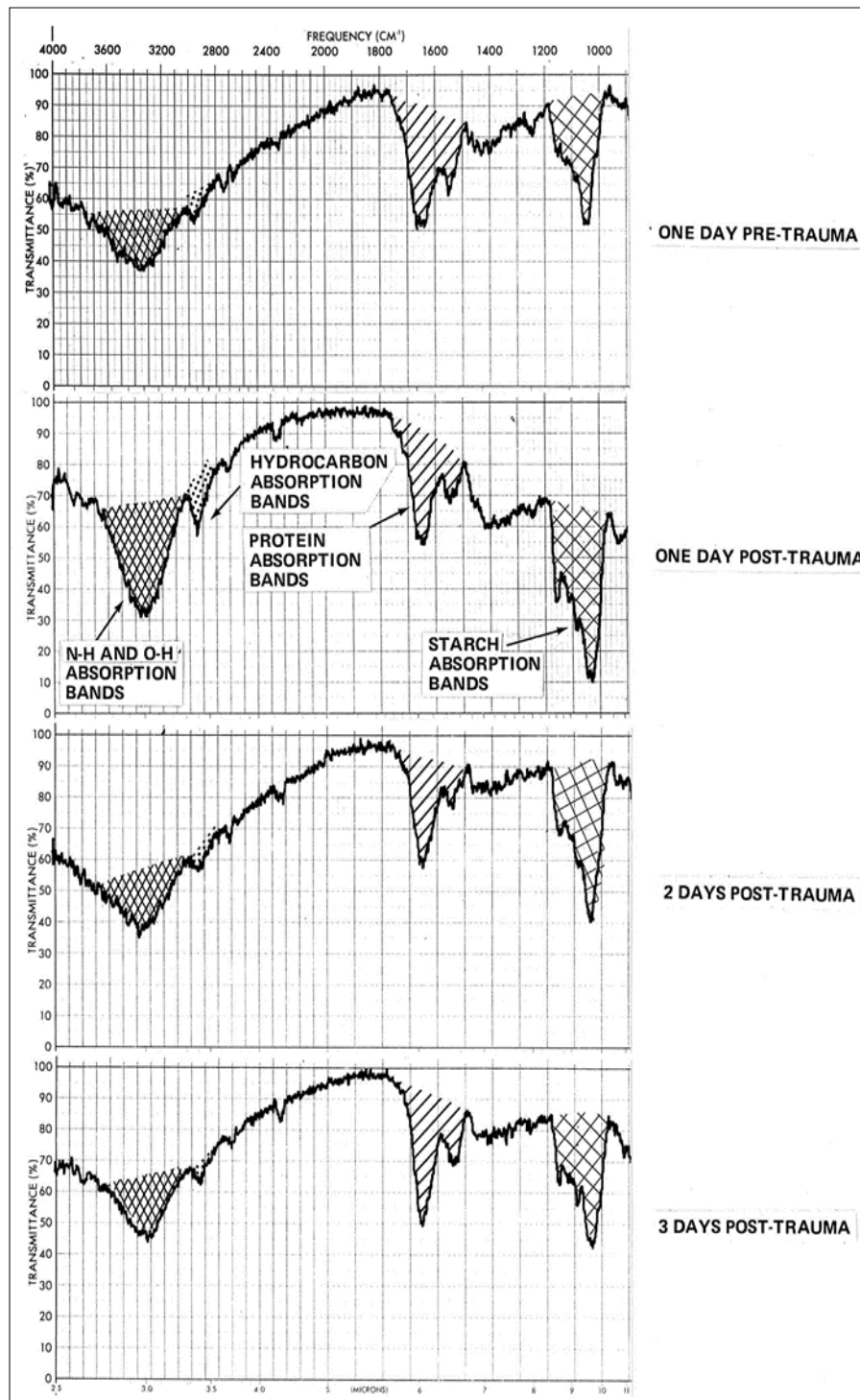


Figure 5 - Infrared (IR) spectral records showing the anomalous and rapid shift in protein-to-starch ratio in mucin following person's physical trauma (car accident) and accepting medication.



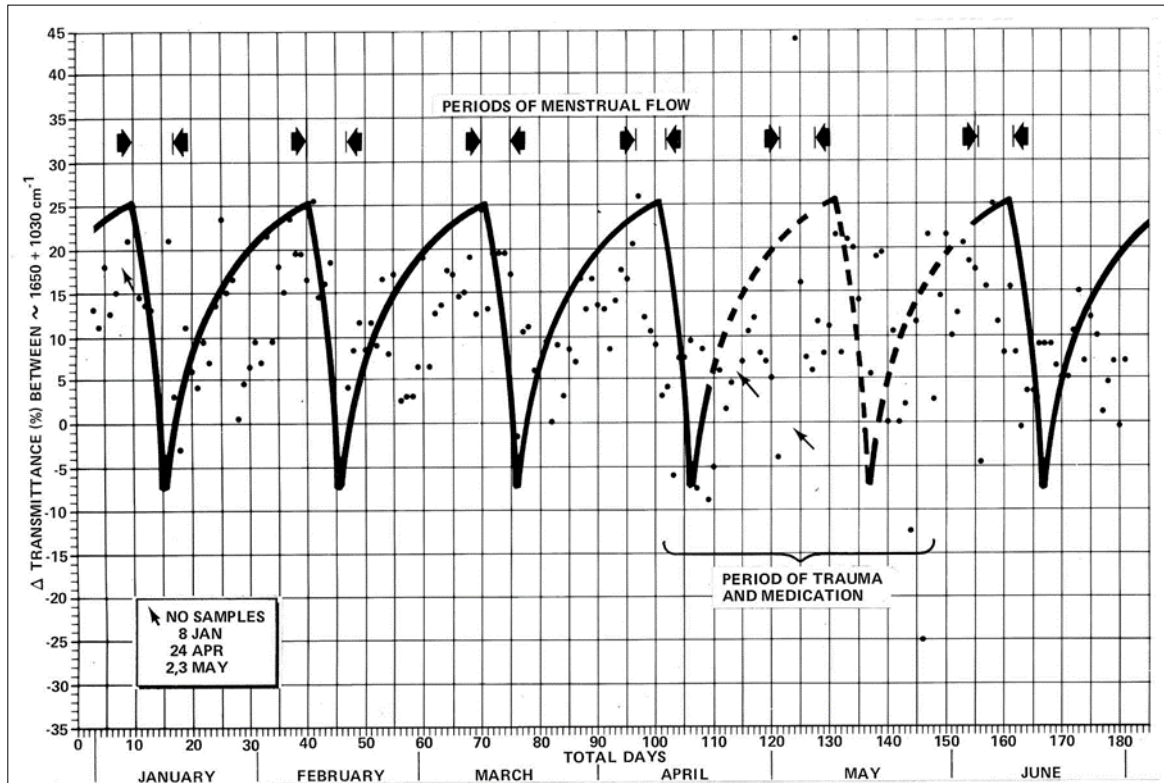


Figure 6 - An example of the poor correlation of the expected 30-day cycle with actual mucin IR absorption data showing the variable protein-to-starch ratios. Simple checking of the calendar will not be sufficient to choose the actually most-fertile period.

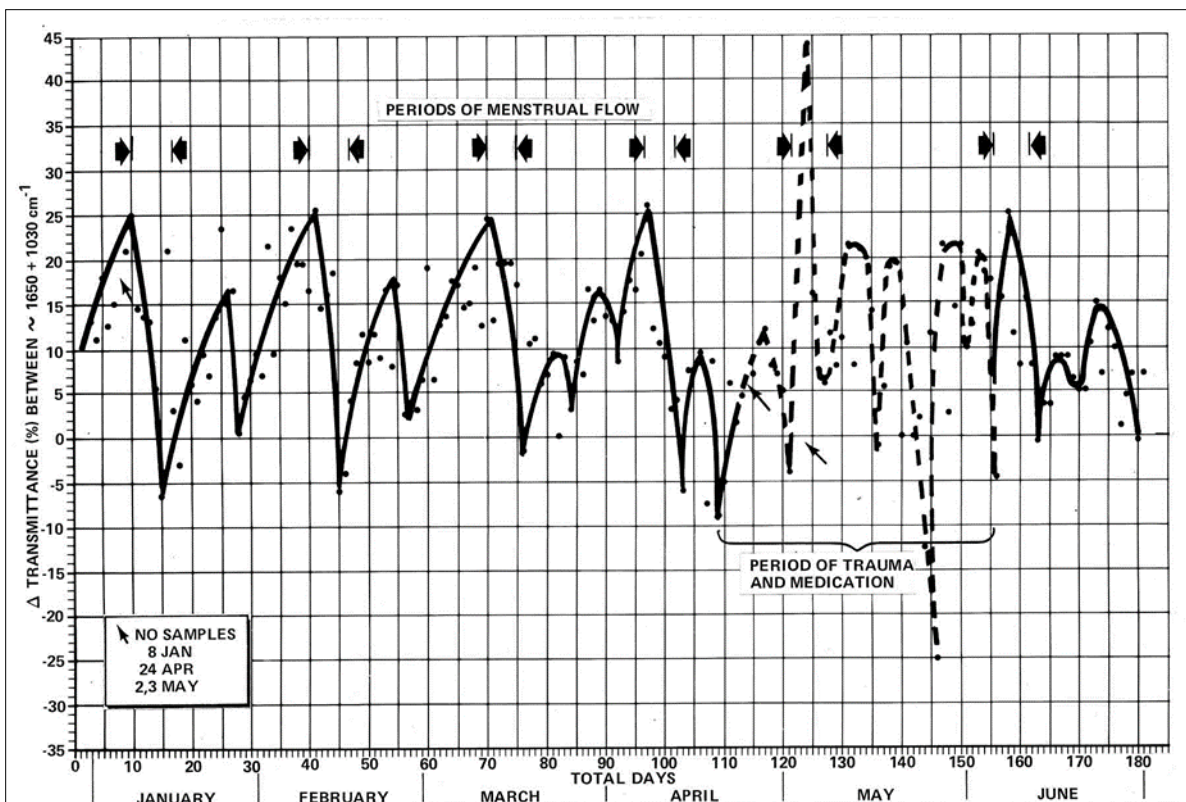


Figure 7 - An improved correlation of vaginal mucin protein-to-starch ratios occurs when variations are examined over 12-14 day cycles.

be used to overcome the impaired "gate-keeper" function of the cervical mucus plug in infertility cases.<sup>32,33</sup>

### Conclusion

The expression of variable mucin core proteins and their variable length glycans represents a key feature demonstrated to vary with the fertility cycle. It is, thus, quite likely that the simple objective measure of protein-to-carbohydrate ratios for mucin glycoproteins will be predictive of the chemistry-dictated mechanics of these substances still being interrogated by increasingly sophisticated techniques.<sup>34, 35</sup>

### Acknowledgments

Ms. S. Perlmutter and Mrs. C. George recorded the vast majority of infrared spectral traces required for this investigation, and performed most of the instrument calibration studies. C.P. Cozad, H. Hammill, and R. Zontek of Calspan Advanced Technology Center provided computational help, under internally funded research project #6234-M. Professor P-O Glantz, Malmo University, Sweden, supervised concurrent oral adhesion and saliva studies. OB-GYN SUNY Buffalo Faculty Dr. Ronald Batt and Dr. John Naples provided significant fertility-related clinical data.

Protocol and Consent Approvals: At Calspan Advanced Technology Center, December 7, 1977, Approved by Human Subjects Committee review via Procedure 309. At State University of New York at Buffalo (SUNY), November 6, 2008 approval was given by the Health Sciences Institutional Review Board as HSIRB # SIS0531008E.

### Conflict of Interest

The authors report no conflict of interest concerns with this study.

### Supplementary Data

Data Plotting and Reduction Techniques

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

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