# Menstrual cycle–related sialidase activity of the female cervical mucus is associated with exosome-like vesicles

*Federica Flori, B.Sc.,*[a] *Federica Secciani, Ph.D.,*[a] *Antonietta Capone, Ph.D.,*[a] *Eugenio Paccagnini, B.Sc.,*[a] *Sabrina Caruso, B.Sc.,*[b] *Maria Grazia Ricci, M.D.,*[b] *and Riccardo Focarelli, Ph.D.*[a]

[a] Department of Evolutionary Biology, University of Siena, and [b] Department of Obstetrics and Reproductive Medicine, University of Siena, Policlinico le Scotte, Siena, Italy

**Objective:** To study endogenous sialidase activity in genital tract secretions of pregnant and nonpregnant women.
**Design:** Laboratory study.
**Setting:** Department of Evolutionary Biology and Department of Obstetrics and Reproductive Medicine, University of Siena, Siena, Italy.
**Intervention(s):** Vaginal and cervical mucus samples were obtained from pregnant and nonpregnant women in different phases of the menstrual cycle and in different weeks of pregnancy.
**Main Outcome Measure(s):** Sialidase activity was assessed by fluorimetric assay and localized by transmission electron microscopy and differential centrifugation.
**Result(s):** Sialidase activity in cervical mucus of healthy women reaches a maximum in the ovulatory phase. Cervical mucus from pregnant and nonpregnant women had significant sialidase activity that was associated with membranous vesicles having an exosome-like structure.
**Conclusion(s):** Female cervical mucus contains an endogenous menstrual cycle–related sialidase that could be involved in modifying the rheologic properties of mucus to favor sperm progression at fertilization. Its association with exosome-like vesicles also suggests a role in intercellular communication before and after fertilization. (Fertil Steril® 2007;88(Suppl 2):1212–9. ©2007 by American Society for Reproductive Medicine.)

**Key Words:** Cervical mucus, exosome, menstrual cycle, pregnancy, sialidase

Carbohydrate-based interactions have been described in different aspects of the fertilization process (1, 2). The rheologic properties of uterine and cervical mucus have been suggested to play a role in sperm progression. Several lines of evidence indicate that the glycoproteic meshwork of cervical mucus and its ability to allow sperm migration of spermatozoa significantly change during the menstrual cycle, irrespective of transcriptional activity of female genital tract epithelia (3, 4). Female mucus consists largely of mucins, heavily glycosylated proteins, which are secreted by different epithelial cells of the female genital tract. In humans, seven mucin genes are expressed by endocervical epithelial cells, namely MUC 1, 2, 4, 5AC, 5B, 6, and 8 (5, 6). Although the mucin genes expressed by the epithelia do not change qualitatively during the menstrual cycle, quantitative differences have been detected. In particular MUC4 and MUC5B are the prevalent mucin messenger RNAs of the human endocervix (5, 6). MUC5B has its highest expression at midcycle, and its oligosaccharide moieties show similar cycle-dependent structural modifications (6, 7). These results support the hypothesis that changes in rheologic and physiologic properties are not linked to the mucin protein backbones but may reside in their carbohydrate moieties. Such speculation has been supported by studies in humans (3) and in the bonnet monkey *Macaca radiata* (8). In particular, Nasir-ud-Din et al. (8) indirectly demonstrated that negatively charged groups in the oligosaccharide moieties of mucins, that is, sialic acid and/or sulfate groups, are fundamental in maintaining the structural and functional integrity of mucus glycoproteins.

Sialidases are a family of exoglycosidases, the presence of which was reported more than 20 years ago in secretions of the female genital tract (9). These authors described cyclic changes in specific sialidase activity of cervical mucus toward endogenous substrates. This enzyme activity was then studied for its role as a virulence factor in pathologic conditions such as bacterial vaginosis (10–12). Sialidase activity was therefore considered for the development of a diagnostic test for bacterial vaginosis (12, 13). However, most of these studies did not assess quantitative parameters, such as the whole protein content of samples or women's ages or the

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These authors contributed equally to this research.
Reprint requests: Riccardo Focarelli, Ph.D., Department of Evolutionary Biology, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy (FAX: 0039-577-234476; E-mail: focarelli@unisi.it).


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period of the menstrual cycle in which the mucus samples were obtained. For obvious reasons, they mainly addressed the enzyme activity present in vaginal secretions.

In this article we report the results of comparative analysis of endogenous sialidase activity in cervical mucus of women during the menstrual cycle and pregnancy. Localization of the activity on exosome-like vesicles is reported, and its presence in vaginal mucus was also investigated.

## MATERIALS AND METHODS

### Sampling and Sample Preparation

Vaginal and cervical mucus samples were obtained from a total of 158 women. The women were between 17 and 45 years of age. They had normal menstrual cycles, were not using intrauterine devices or oral contraceptives, and were without infections (see below), inflammatory diseases, or endometriosis. The menstrual cycle was determined according to cycle history with day 1 defined as the first day of fresh red bleeding. The cycle was thus divided into three phases: preovulatory phase (from day 5 to day 11), ovulatory phase (from day 12 to day 16), and luteal phase (from day 17 to day 29). Cervical mucus samples were also collected from 150 women in weeks 27 to 39 of pregnancy. Samples were collected by scraping the vaginal mucosa and endocervical canal with sterile cotton swabs. All women were recruited during routine gynecologic examination and gave their informed consent to the study. The study protocol was approved by the ethical committee of Siena University.

During gynecologic examination, samples from each woman were also evaluated for bacterial vaginosis, defined as the presence of at least three of the four criteria described by Amsel (14): [1] atypical vaginal discharge with homogeneous adherence to the vaginal walls, [2] vaginal pH >4.5, [3] sniff test positivity, and [4] microscopic evidence of clue cells with or without Gram staining. Bacterial vaginosis was diagnosed in nine women. Their sialidase content was determined, but these values were excluded from the study.

Vaginal and cervical swabs were immediately placed in 500 $\mu$L of 400 mmol/L sodium acetate buffer pH 4.2 containing 20 $\mu$mol/L aminoethylbenzenesulfonylfluoride, 1.3 mmol/L bestatin, 0.14 mmol/L *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64), 0.01 mmol/L leupeptin, and 0.003 mmol/L aprotinin (protease inhibitor cocktail; Sigma-Aldrich, St. Louis, MO).

### Assay of Sialidase Activity

Sialidase specific activity was determined with a fluorometric assay using 4-methylumbelliferyl-N-acetyl-$\alpha$-D-neuramide (Sigma-Aldrich) as substrate. In brief, a 25 $\mu$L aliquot of sample was incubated with 25 $\mu$L of 2 mmol/L substrate in sodium acetate 400 mmol/L, pH 4.2, at 37°C for 60 minutes. After incubation, 500 $\mu$L of 85 mmol/L glycine/sodium carbonate buffer, pH 10.0, was added to stop the reaction, and samples were centrifuged at 12,500 $\times$ $g$ for 3 minutes.

The amount of fluorescent product generated in the reaction was measured in a series 1100 fluorimeter (Agilent Technologies, Waldbronn, Germany) in a high-performance liquid chromatography system (Agilent Technologies) at a flow rate of 0.5 mL/min of $H_2O$, using excitation light at 365 nm and emission wavelength of 448 nm.

All assays were performed in triplicate with heat-inactivated samples (100°C for 5 minutes) and samples without substrate as control. The standard curve of the assay was created with use of known amounts of umbelliferone (Sigma-Aldrich), and specific sialidase activity was defined in nanomoles of 4-methylumbelliferone formed per microgram of protein per minute. Total protein concentration was determined by bicinchoninic acid assay (Sigma-Aldrich) with use of bovine serum albumin as standard.

Specificity for sialidase was demonstrated by inhibiting enzyme activity with 100 mmol/L 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Sigma-Aldrich). The specific activity obtained in the presence of the inhibitor was less than 10% of control samples. In some cases sialidase activity was determined in the presence of 5 mmol/L ethylenediaminetetraacetic acid (EDTA).

### Localization of Sialidase Activity

To determine whether the enzyme was present in a soluble or cell-bound form, we sequentially centrifuged two pooled cervical mucus samples from women who were not pregnant (n = 21) and pregnant women (n = 34). In brief, cells were removed by centrifuging for 20 minutes at 2000 $\times$ $g$. Supernatants were collected and centrifuged for 30 minutes at 10,000 $\times$ $g$. Supernatants were collected again and centrifuged for 60 minutes at 100,000 $\times$ $g$ in a SW65 rotor (Beckman Instruments, Inc., Fullerton, CA). All the above steps were performed at 4°C. At the end of each run sialidase activity was determined in pellets and supernatants as described above. The final pellet was then resuspended in phosphate-buffered saline solution (50 mmol/L $KH_2PO_4$, 150 mmol/L NaCl, pH 7.4) and processed for transmission electron microscopy or stored at −80°C.

### Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western Blotting

Proteins of the final pellet obtained by differential centrifugation were loaded on a 12% polyacrylamide gel according to Laemmli (15). After electrophoresis, proteins were silver stained (see below) or transferred to a nitrocellulose membrane according to Towbin et al. (16). The sheets were blocked with 3% (wt/vol) nonfat powdered milk in tris(hydroxymethyl)aminomethane (Tris)-buffered saline solution (TBS; 20 mmol/L Tris-HCl, 500 mmol/L NaCl, pH 7.5) and incubated for 1 hour at room temperature with a polyclonal anti-$\beta$ tubulin or a monoclonal antiactin antibody

(Sigma-Aldrich) at dilutions of 1:1,000 and 1:250. After several washings with TBS-Tween 20 0.2% (vol/vol) (TTBS), blots were incubated for 1 hour with the corresponding secondary antibody conjugated with alkaline phosphatase or peroxidase (BioRad Microscience, Cambridge, MA). After extensive rinsing in TTBS, the reactions were developed by using the Immun-star chemiluminescent protein detection system (BioRad Microscience) according to the manufacturer's instruction. Chemiluminescence was visualized with a Chemi DOC XRS system (BioRad Microscience).

### Two-dimensional Gel Electrophoresis

For two-dimensional gel electrophoresis, proteins were first precipitated with chloroform/methanol according to Wessel and Flugge (17) and resuspended in distilled water for determination of protein content. A 24 $\mu$g aliquot was lyophilized and resuspended in a rehydration buffer containing 8 mol/L urea, 2 mol/L thiourea, 4% (wt/vol) 3[3-cholaminopropyl diethylammonio]-1-propane sulfonate, 65 mmol/L dithiothreitol (DTT), 1.5% (vol/vol) immobilized pH gradient (IPG) buffer pH 3–10, and 0.001% (wt/vol) bromphenol blue. Isoelectric focusing was carried out on a nonlinear wide range of immobilized pH gradients (pH 3–10; 18-cm-long immobilized pH gradient strips) with use of the IPGphor system (Amersham Biosciences, Uppsala, Sweden) for a total of 80,000 Volts/hour. Focused strips were first equilibrated for 12 minutes at room temperature in 50 mmol/L Tris-HCl pH 6.8, 6 mol/L urea, 30% (vol/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate (SDS), and 1% (wt/vol) DTT and then for 5 minutes in the same buffer in which DTT was replaced by 2.5% (wt/vol) iodoacetamide. The second dimension was carried out on a 12% polyacrylamide gel (18 × 20 × 1.5 mm) and silver stained according to Blum et al. (18) with minor modifications. Stained gel was acquired by using a Chemi-Doc XRS system (BioRad Microscience). Image analysis was carried out by using PDQuest software (BioRad Microscience).

### Transmission Electron Microscopy

Pellets obtained after 100,000 × *g* centrifugation were fixed in 2% paraformaldehyde, loaded on Formvar/carbon-coated grids, postfixed in 1% osmium tetroxide, and contrasted successively in 2% uranyl acetate, pH 7.0. Observations were made with a Philips CM10 electron microscope (Eindhoven, The Netherlands).

### Statistics

Specific enzyme activity was analyzed by one-way ANOVA in cervical mucus samples of pregnant women and in vaginal and cervical mucus samples of nonpregnant women. The factor was menstrual cycle phase (three levels: preovulatory phase, ovulatory phase, luteal phase). A Duncan multiple-range test at $P<.05$ was used to locate significant differences identified by one-way ANOVA. The Statistica package (StatSoft, Tulsa, OK) was used for statistical analysis.

## RESULTS

### Sialidase Activity in Female Genital Tract

In a first series of experiments we evaluated the specific sialidase activity of vaginal and cervical mucus in 158 women grouped according to phase of the menstrual cycle. None of the women had bacterial vaginosis. The results are shown in Figure 1 and Table 1.

An active enzyme with an optimum pH around 4.2 (data not shown) was found in both types of mucus in almost all women, with a peak of activity in ovulatory phase. One-way ANOVA detected significant differences in cervical mucus enzyme activity values ($F_{(2,51)} = 5.67$; $P<.01$). The Duncan multiple-range test detected significant differences in mean values between ovulatory and the other two menstrual cycle phases ($P<.05$). On the contrary, no significant differences could be detected by one-way ANOVA for the enzyme activity of the vaginal mucus samples ($F_{(2,57)} = 0.7$; $P=$ not significant [NS]).

Mean specific sialidase activity of vaginal mucus was 0.036 nmol/$\mu$g/min versus 0.16 nmol/$\mu$g/min for cervical mucus. This difference is almost entirely due to the lower median protein content of cervical mucus (0.283 mg/mL) with respect to vaginal samples (1.813 mg/mL). Furthermore, the two enzymes appeared to differ in certain biochemical properties, such as their dependence on bivalent ions for activity, suggesting an unknown extent of bacterial contamination (data not shown). We therefore decided to focus mainly on the enzyme of cervical mucus.

At this point we decided to investigate the presence of sialidase activity in the mucus of pregnant women. As shown in

**FIGURE 1**

Specific sialidase activity in cervical mucus of women in different phases of the menstrual cycle: preovulatory phase (*pre-OP*), ovulatory phase (*OP*), and luteal phase (*LP*). Specific activity is expressed in nanomoles of 4-methylumbelliferone formed per microgram of protein per minute.

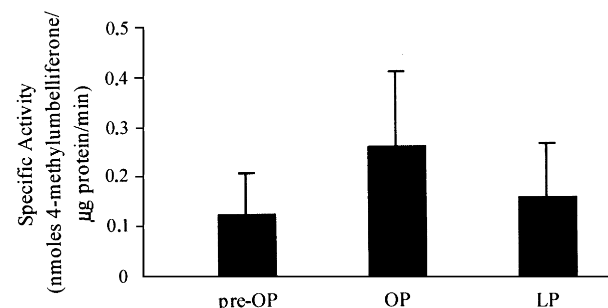


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**TABLE 1**

**Specific sialidase activity, defined as nanomoles of 4-methylumbelliferone formed per microgram of protein per minute in cervical and vaginal mucus of women in different phases of the menstrual cycle.**

| Phase | Cervical specific activity (nmol 4-methylumbelliferone/μg protein/min) | Vaginal specific activity (nmol 4-methylumbelliferone/μg protein/min) |
|---|---|---|
| Preovulatory | | |
| n = 15 | 0.122 ± 0.084 | |
| n = 18 | | 0.033 ± 0.049 |
| Ovulatory | | |
| n = 23 | 0.258 ± 0.148 | |
| n = 25 | | 0.043 ± 0.042 |
| Luteal | | |
| n = 36 | 0.153 ± 0.109 | |
| n = 41 | | 0.030 ± 0.026 |

*Note:* Fluorimetric assay was performed at pH 4.2 with use of 25 μL cervical and vaginal mucus and 4-methylumbelliferyl-N-acetyl α-D-neuramide as substrate. Results are expressed as mean for experiment ± SD.

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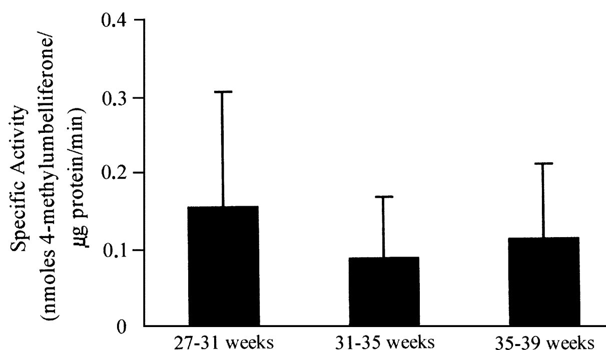
Figure 2, sialidase activity was present in the cervical mucus of pregnant women at the end of gestation with quantitative levels similar to those of nonpregnant women. The specific activity did not undergo significant modifications in the last 3 months of pregnancy as determined by one-way ANOVA ($F_{(2, 24)} = 0.7919$; $P$=NS).

### Localization of Sialidase Activity in Cervical Mucus

To determine whether sialidase was soluble or cell bound, we subjected pooled samples of cervical mucus of pregnant and nonpregnant women to differential centrifugation and assayed the sialidase activity in pellets and supernatant. In both samples, activity was always found in supernatant after low-speed centrifugation and in pellets after high-speed centrifugation (100,000 × *g*) (Table 2). Because identical patterns were found in samples of nonpregnant and pregnant women, we used cervical mucus samples from pregnant women for the following experiments, because routine examination during pregnancy made it more available. Whole mount preparations of high-speed pellets were viewed by transmission electron microscopy after staining with osmium tetroxide and uranyl acetate. As shown in Figure 3, small membranous vesicles with a diameter in the range of 100 to 200 nm were detected. They resembled the exosome-like vesicles secreted by several different cell types (19, 20).

**FIGURE 2**

Specific sialidase activity in cervical mucus of pregnant women at different weeks of gestation defined as nanomoles of 4-methylumbelliferone formed per microgram of protein per minute.

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To define the nature of these vesicles we separated their protein content by SDS–polyacrylamide gel electrophoresis (PAGE), blotted it onto nitrocellulose membranes, and probed it with antibodies against β-tubulin and actin, two structural proteins known to be present in most, if not all, the exosome vesicles so far characterized. As shown in Figure 4, the membranes were positive for both antibodies. However, the antiactin antibody revealed two bands of similar molecular weight, suggesting the presence of at least two isoforms in the vesicles. Another cytoskeleton-associated protein, ezrin, also reported in exosomes, was identified by mass spectrometry of the band excised from the silver-stained gel after trypsin digestion (Fig. 4). This analysis was performed at CNR-ISPA (Turin, Italy). As a preliminary step for complete analysis of the mucus exosomes, two-dimensional electrophoretic separation was also performed. The protein pattern (Fig. 5) contained about 180 protein spots.

## DISCUSSION

Sialic acids are a very heterogeneous group of nine-carbon sugars, common as terminal nonreducing components of oligosaccharides in glycoproteins and glycolipids. Because

**TABLE 2**

**Localization of sialidase activity in pooled samples of cervical mucus from pregnant women.**

| Step | Total protein (μg) | Total activity (unit) | Specific activity (unit/μg) |
|---|---|---|---|
| Pool | 18,000.00 | 1,800.00 | 0.100 |
| Supernatant (2,000 × *g*) | 9,960.00 | 1,205.16 | 0.121 |
| Pellet (2,000 × *g*) | 1,021.90 | 20.44 | 0.020 |
| Supernatant (10,000 × *g*) | 8,032.32 | 578.33 | 0.072 |
| Pellet (10,000 × *g*) | 120.00 | 0.36 | 0.003 |
| Supernatant (100,000 × *g*) | 6,240.00 | 56.16 | 0.009 |
| Pellet (100,000 × *g*) | 168.00 | 10.75 | 0.064 |

*Note:* Sialidase activity and protein content in supernatant and pellets after low-speed centrifuging at 2,000 × *g* and 10,000 × g and then after high-speed centrifuging (100,000 × g for 60 minutes).

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of their negative charge, they often strongly influence the three-dimensional structure of proteins and extracellular matrices, as well as cell-cell interactions (21). It therefore is not surprising that sialidase activity has been viewed as a potential regulator of various biologic processes, although, in most cases, the physiologic significance of desialylation is largely unknown because of the incomplete knowledge of the structure and function of mammalian sialidases (22, 23).

**FIGURE 3**

Transmission electron micrographs of purified exosomes from cervical mucus of pregnant women obtained by high-speed centrifugation (100,000 × *g*). Note membrane vesicles approximately 100 to 200 nm in diameter; scale bars = 100 nm.

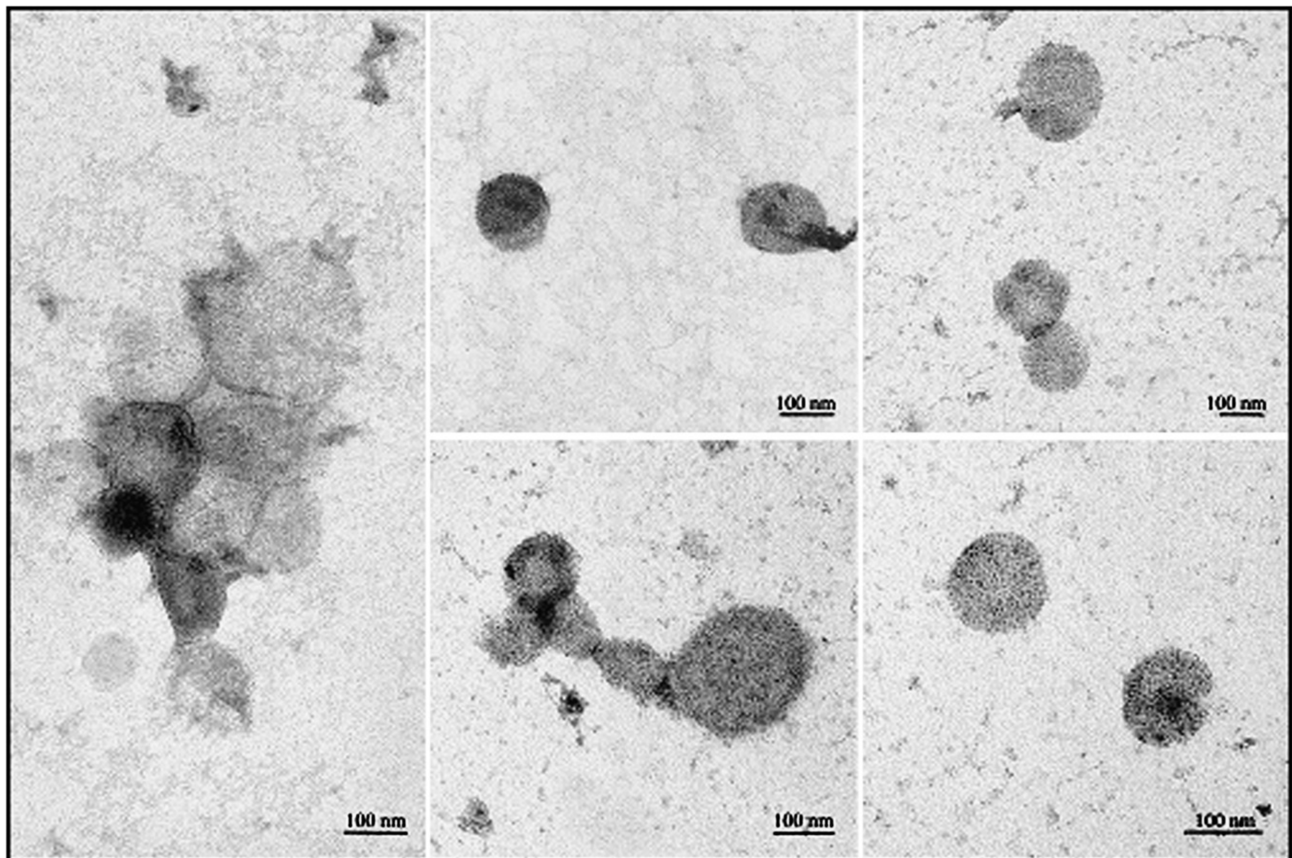


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FIGURE 4

Electrophoretic analysis of membrane vesicles obtained by high-speed centrifugation (100,000 × *g*) of cervical mucus of pregnant women. Proteins separated in 12% SDS-PAGE gel and visualized by silver staining (*1*), or probed with antiactin (*2*) and antitubulin (*3*) antibodies. *Arrow* indicates band corresponding to ezrin.

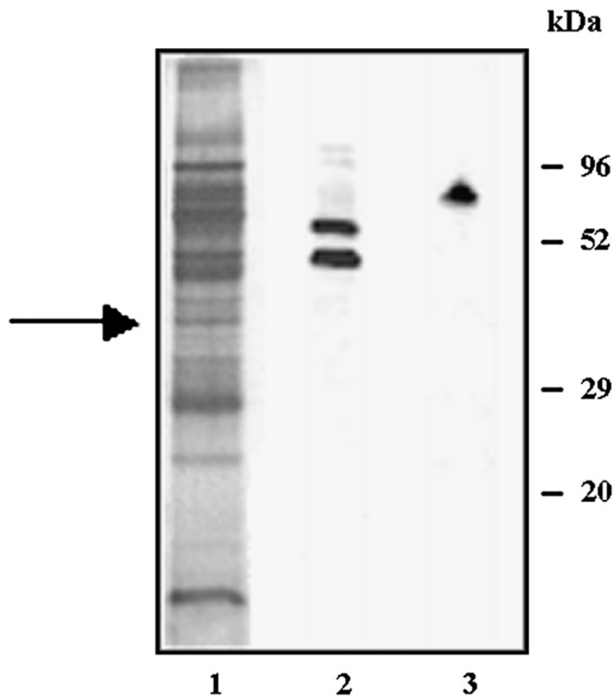


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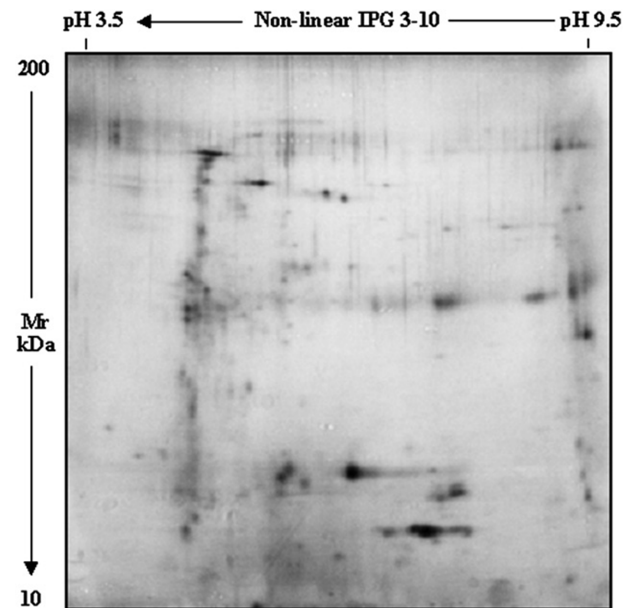
FIGURE 5

Silver-stained two-dimensional electrophoretic separation of membrane vesicles obtained from cervical mucus of pregnant women by high-speed centrifugation (100,000 × *g*).

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Sialic acids are often evoked as modulators of key steps in the mammalian fertilization process. For example, we previously demonstrated that sialylglycoconjugates are removed from the human sperm surface during in vitro capacitation (24), and our results were confirmed by observation that a sialic acid–binding lectin in human endometrial cells induced sperm capacitation (25).

Several studies have clearly demonstrated that changes in human cervical mucus during the menstrual cycle strongly influence sperm orientation and migration (3, 4) and possibly sperm physiology (see for example Zinaman et al. [26]). It is well known that changes in the rheologic properties of cervical mucus are due to modifications in cervical mucins, especially their carbohydrate composition. It is interesting that sulfate groups and sialic acids (i.e., charged molecules) were recently reported to play a fundamental role in the physiologic functions of cervical mucus in bonnet monkeys, *Macaca radiata* (8). Thus the presence of sialic acid–modifying enzymes, such as sialidases, in cervical mucus could be an important factor for the correct development of sperm physiologic functions in the context of the whole reproductive process. On the other hand, the presence of sialidase activity in vaginal fluids has often been regarded as an important marker of infectious diseases that could impair female reproductive capacity.

Using a highly sensitive fluorimetric assay, we demonstrated endogenous sialidase activity in the female genital tract of pregnant and nonpregnant women. Its activity in cervical mucus reached a maximum in the ovulatory phase of the menstrual cycle, thus confirming and quantitatively expanding the results of Paulesu and Pessina (9). Furthermore, statistical analysis detected significant differences between menstrual cycle phases with a clear trend toward an increase in sialidase activity at the ovulatory phase. These results suggest that an increase in sialidase activity could play a role in modifying the rheologic and ultrastructural properties of cervical mucus, thus facilitating sperm progression and/or capacitation in the female genital tract. We also found a similar, though not statistically significant, pattern for the vaginal mucus enzyme.

Another difference between vaginal and cervical mucus was that the activity of the vaginal enzyme was almost completely abolished by 5 mmol/L EDTA in the medium whereas the cervical enzyme activity was only partially affected. Because calcium ion dependence more often has been associated with bacterial sialidases than with mammalian ones, we suspected a partial exogenous origin of the sialidase of

vaginal mucus that may also account for the lack of statistical significance of its values.

Hence, individual parameters not addressed in this study, such as intercourse frequency and douching, may significantly affect vaginal mucus sialidase activity levels. Further biochemical characterization of the two enzymes is needed to assess this point.

Furthermore, our results also showed that the level of specific sialidase activity was consistently lower in vaginal mucus than in cervical samples mainly because of the lower median protein content of cervical mucus samples. This indicates the importance of a quantitative approach for interpreting the possible clinical consequences of sialidase activity in the female genital tract. In fact, by the simple criterion of the emitted luminescence units (LU), sialidase activity would be higher in vaginal mucus (2.8 LU) than in cervical samples (2.3 LU). This is why accurate and repeated determination of specific sialidase activity seems to be necessary for any diagnostic approach.

Interestingly, significant sialidase activity was also detected in cervical mucus during pregnancy. Sialidase activity in the female genital tract during pregnancy has already been found in studies seeking correlations between high enzyme activity, as indicator of bacterial infections, and preterm births (27, 28).

Using differential centrifugation and transmission electron microscopy we also demonstrated that the cervical mucus enzyme occurred in membrane-bound form on membranous vesicles, resembling the exosomes already reported to be secreted by several cell types. This observation has also been confirmed by preliminary demonstration of cytoskeletal proteins, such as ezrin (a cross-linker of actin to plasma membrane), $\beta$-tubulin, and actin, which are common constituents of these vesicles in other cell types (19, 20). Although the definition of exosomes and their functions is not yet unanimous (29), a possible role in intercellular communication and/or immune response modulation in the female genital tract should be considered for future studies.

As already reported, the lability and low-level expression of mammalian sialidase severely hampers a classic biochemical approach to its characterization. For a better understanding of the function and the cellular source of cervical mucus sialidase, cloning of its gene would therefore be appropriate.

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