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Morphological characterization of different human cervical mucus types using light and scanning electron microscopy

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BACKGROUND: This study was conducted on human cervical mucus using light microscopy (LM) and scanning electron microscopy (SEM). The objective was the morphological characterization of the different mucus types, with samples taken from the lumen of the cervix and from the different secretory zones of the cervical mucosa. METHODS: A total of 230 samples from 195 women were spread out on slides and air dried. The phenomenon of 'ferning' was observed and assessed in these samples using both LM and SEM. Further samples from the lumen of the cervix and the different secretory crypts were spread out on cover slips and fixed with glutaraldehyde (2.5%) to be studied by SEM. RESULTS: The results show the presence of four different morphological mucus types, namely L, S, P and G, in both types of sample using dried and fixed techniques. CONCLUSIONS: Mucus from the lumen of the cervix appears to be a morphologically heterogeneous entity. It contains different types of secretions, the proportions of which vary throughout the menstrual cycle. The different mucosal types show different types of crystallization, different patterns of ultrastructure (probably related to the arrangement of the glycoprotein network) and are produced in different secretory zones of the crypts in the cervix.

Key words: cervical mucus/ferning/light microscopy/morphology/scanning electron microscopy

Introduction

Cervical mucus is a hydrogel produced by the cervical glands (Elstein *et al*., 1973; Odeblad, 1973). It is involved in sperm migration and maturation through the female genital tract, and provides a barrier to prevent the pathogens entering the endometrium. It is also related to pathology of the cervical immune system (Ginsburg *et al*., 1997).

This mucus changes with the menstrual cycle, which means it has different biophysical and biochemical characteristics throughout the cycle (Elstein, 1978). Mucus is therefore an indirect but important element for estimating the time of ovulation, not only for clinicians but also for women using natural family planning methods, especially the Billings method (Billings and Westmore, 1992; Ryder and Campbell, 1995; Parrilla and Delgado, 1997).

Nowadays, it is generally accepted that cervical mucus is not a homogeneous entity, but instead a heterogeneous one, containing different types of secretions that vary in proportion throughout the cycle (Odeblad *et al*., 1983; Ryder and Campbell, 1995; Odeblad, 1997). These different units or mucus types have been characterized by both nuclear magnetic resonance (NMR) and light microscopy (LM) techniques (Odeblad *et al*., 1983). Although they have not yet been characterized as different units from a biochemical point of view, there is biophysical and morphological evidence of this heterogeneity. Our biophysical evidence is based on the different viscosities of the different units. The morphological evidence is based on the 'ferning' phenomenon observed in dried mucus samples when spread out on a slide in all directions (Odeblad *et al*., 1983).

Previous studies indicate that human cervical mucus contains four different types of secretion, namely G (gestagenic), L (loaf), S (string) and P (peak) mucus, each with a different viscosity (NMR studies) and a different morphology in the crystals of air-dried mucus samples (Odeblad, 1997). There are observations which suggest that these four mucus types are produced in specific crypts in different areas of the cervix (Odeblad, 1966; 1994a, 1997). All this information could lead to important changes in the study of human cervical mucus and add new knowledge. It also means that we would need further research to complement our knowledge of the nature of cervical mucus.

Scanning electron microscopy (SEM) is an important technique used in recent years to study cervical mucus (Chrétien, 1973; Chrétien *et al*., 1973; Barros *et al*., 1985; Vigil *et al*., 1991). It has enabled us to study the relationship between mucus molecular architecture and mucus function using fixing techniques (Chrétien *et al*., 1973; Daunter, 1984;

Table I. The ranges of values for each mucus type and phase of the cycle

Mucus type	Early estrogenic phase, day –12 to –6 (%)	Late estrogenic phase, day –5 to –3 (%)	Early ovulatory phase, day –2 to 0 (%)	Late ovulatory phase, day +1 to +2 (%)	Gestagenic phase, day +3 to +12 (%)
L	4–10	20–50	50–80	10–6	2–6
S	0–2	8–18	18–38	12–8	0.5–1
P	0–1	2–4	1–4	1–5	0.5–3
G	75–100	20–50	0–5	65–85	85–100

The mucus samples studied are divided into early estrogenic phase (from day –12 to –6), late estrogenic phase (from day –5 to –3), early ovulatory phase (from day –2 to 0), late ovulatory phase (from day +1 to +2) and gestagenic phase (from day +3 to +12), taking 0 as the day of ovulation. The mean age of the women in our study is 30 years (range 24–36).

Barros *et al*., 1985). Furthermore, SEM has made characterization of dried mucus easier (Zaneveld *et al*., 1975), as observations can be made in more detail. However, both study types have been carried out without considering whether mucus from the cervix is a heterogeneous entity made up of different units.

The aim of this study is to provide new morphological information to confirm or reject the idea of the existence of four diverse mucus types and to determine whether the molecular network architecture is different in the four mucus types. This would help us to better characterize each one from a morphological point of view and to give us new evidence of their different properties.

To attain these two objectives, we performed three different but complementary studies. (i) We wanted to know whether the mucus types from specific crypts was similar to those from the cervical canal. To determine this, we took samples from the two locations, spread them out and air-dried them for observation by LM and SEM. (ii) After verifying that the mucus types from the crypts were similar to those from the canal and that each different mucus type came from a different specific crypt zone, we conducted an SEM study on fixed mucus samples from each crypt zone. The aim was to compare the ultrastructures and determine whether there were differences between them that could correspond to a different molecular arrangement in the network. (iii) After finding that each mucus type in the fixed samples corresponded to a different network, we proceeded to identify each of these networks in fixed samples from the cervical canal.

Materials and methods

Samples

In this study, 230 samples were taken at different times of the menstrual cycle from 195 women (mean age 30 years). The samples were taken from the following locations: (i) 180 samples from the lumen of the cervical canal: ten were from the Department of Medical Biophysics, University of Umea, Sweden. The others came from a Family Planning Centre in Alcantarilla, Murcia, Spain. (ii) Fifty samples were taken directly from the zones of the cervical crypts of five women at the Department of Medical Biophysics, University of Umea, Sweden. Ten samples were from the L zone of the cervical crypts, 12 from the S zone, 12 from the P zone and 16 from the G zone.

Collection of cervical mucus

Collection from the lumen of the cervix

The cervix was viewed with a speculum. The cervical orifice was cleaned with dried gauze and then an insulin syringe was inserted, without a needle, into the cervical orifice to obtain samples by aspiration. The mucus was stretched as far as possible with the syringe as it was being deposited on the slide, before being spread out.

Collection from the zones of the cervical crypts

The crypt specimens were taken under colposcopic control with a Pasteur pipette, which had previously been heated over a burner to stretch and deform the end (Odeblad, 1966). We obtained material from the S, L, G and P zones of the cervical crypts. The distribution of crypt zones in the cervix depends on age, number of pregnancies and use of contraception. In a non-pregnant woman, aged 25–30 years and not having used contraception, the cervix averages 22 mm in length and 6 mm in diameter at ovulation. The crypt distribution starting from below and moving upwards is as follows: the G crypts dominate in the lowest 4–5 mm; then there is a zone of L crypts occupying the next 9–10 mm; this is followed by the S zone, for 5–6 mm; and the highest 3–4 mm contains the P crypts. The borderlines between the zones are not clearly defined, as there is some overlap. In this study, samples were taken from the middle part of each zone, and selection was done according to the following criteria: (i) the location of the crypts in the cervical canal; (ii) the cell content; (iii) the ferning pattern; and (iv) the homogeneity of the sample in at least 95% of samples (Odeblad, 1997).

Preparation of the samples

LM: air-dried cervical mucus samples

Most of the sample was spread out on a slide in all directions using a needle (spread-out technique) (Odeblad, 1995). Afterwards, the samples were air-dried at room temperature for at least 15 min before the study.

In the spread-out samples from the cervical canal the mucus was studied with LM. To do so, 10 fields, chosen at random from the sample, were analysed using a 10 × 10 grid in the microscope eyepiece, to note which cervical mucus type was present in each square. The number of squares in the 10 fields for each mucus type was added up and divided by the total number of squares to obtain the percentages of each mucus type.

With this semiquantitative study, the percentages of the different mucus types in each sample were calculated to determine the day of the cycle to which the sample belonged, using the Odeblad model (Odeblad, 1994a; b). All this information is shown in Table I.

In the slides containing dried mucus from the crypts and cervical canal, the different mucus types were studied and photographed. For

this, an Ilford Pan 50 film (UK) and Leitz Dialux (Germany) microscope were used.

SEM: air-dried mucus samples

The dried samples from the middle of the cervix and from the crypts were metallized. This was done with a Polaron Division Bio-Rad model (Newhaven, UK). Then observation was made with Jeol-JSM T 300 and Jeol-JSM 6100 scanning electron microscopes (Tokyo, Japan). The critical point was not established.

SEM: fixed cervical mucus samples

Spreading: the cover slip on which the 'spread-out' (Odeblad, 1995) was done was quickly placed in 2.5% glutaraldehyde cacodylate buffer (0.1 mol/l), to avoid crystallization. After 7 days the glutaraldehyde was eliminated, and we added distilled water avoiding all contact with the air to prevent crystallization, and left the sample for 15 min. We then changed to acetone in the following concentrations: (i) 30 min in 30% acetone; (ii) 30 min in 50% acetone; (iii) 30 min in 70% acetone; and (iv) 30 min in 100% acetone. Afterwards, we dried them by establishing the critical point with a Belzers Union CPD 020 machine (Liechtenstein). The samples were then metallized in a Polaron Division Bio-Rad model and observed with Jeol-JSM T 300 and Jeol-JSM 6100 scanning electron microscopes.

Results

Cervical canal

LM: air-dried mucus samples

We observed the morphology of the different mucus types in samples from the cervical canal and classified them according to Odeblad's criteria (Odeblad, 1997).

L mucus: L mucus had the typical ferning morphology (Figure 1). We observed that it had a structure with a straight or curved central axis and branches protruding from it at a 90° angle. These branches could also act as an axis for new branches, again at a 90° angle. On observing the details, we noted crystals on the axis and branches.

S mucus (Figure 2): S mucus had three subtypes: S1, S2 and S3. Subtype S1 consisted of a parallel arrangement of crystals that were close together but not joined. The S2 subtype consisted of a parallel arrangement, but this time the crystals were joined, without branches. In the S3 subtype, we observed a parallel arrangement of crystals with short branches protruding from them. We could not see the organic substrate under the crystals in any of the subtypes.

G mucus: G mucus had a high free-crystal content with no predetermined form (Figure 3). In some cases it had a high cell content.

P mucus: we found five different subtypes for P mucus. Subtype P6B: this subtype presented a star-like morphology with six well-defined axes (hexagonal symmetry). There were branches protruding from the axes at a 60° angle. Subtype Pa: this subtype presented a star-like morphology, without axes, with radial symmetry. Branches coming from the centre had a 60° angle. Subtype P2: this consisted of a central axis with branches at a 60° angle on both sides of the axis. At times this main axis was divided into two, and again into two more, although always at a 60° angle. Subtype 4: the ferning had two fundamental and well-defined axes, with a 90° angle between them. Branches projected from the main axis at a 60° angle (Figure 4). Subtype Pt: the morphology of the branches coming from the main axis consisted of triangular crystals. Both axis and branches seemed to be formed by crystals, which were not always joined.

SEM: air-dried mucus samples

SEM enabled us to see in more detail all these air-dried mucus types from the cervical canal.

L mucus: we were able to observe clearly the organic substrate. Branches from the central axis presented two faces. One was straight and the other dentate. We observed crystals inside the ferns (Figure 5). These crystals were hexagonal or cubic and ranged from 5 to 8 μm in size.

S mucus: in some of the S subtypes the organic substrate could be seen. For example, we could see the linear arrangement in the S1 subtype and that the crystals were not joined. In the S2 and S3 subtypes, the arrangement of the crystals was different. For example, the crystals were joined in both cases and had branches in the S3 subtype (Figure 6). The morphology of the crystals was very similar in all the S subtypes and very similar also to those we found in the L mucus. They presented a cubic, hexagonal or polyhedral morphology.

G mucus: the G mucus contained free crystals, with a different morphology and sometimes a large number of cells.

P mucus: we could also see more clearly the characteristics of the different P subtype mucus with SEM. For example, in the P6B subtype we could see the crystallization inside the mucus, in a cubic form, around the axis. The Pa units had different sizes, and we could not identify any axis at all in this subtype. In the P2 samples, the main axis and the branches had 60° angles between them. They had a poorer crystallization. We often found P4 associated with L mucus. In the Pt samples we could see very clearly the crystals with their triangular form. Most of them were rectangular or rounded. The crystallization was very rich.

SEM: fixed mucus samples

The aspect of the mucus from the cervical canal depended on the percentage of each mucus type in the samples. It was possible to observe four different structural organizations in the cervical mucus.

Type I: compact mucus, with small pores (0.2–0.4 μm in diameter). This was very common in the samples with a high percentage of G mucus (60–94%). It frequently presented large plain surfaces (Figure 7).

Type II: this was not as compact as the type described above. The structure was similar to that of a marine sponge, with diameters of 0.4–1.3 μm. It was common in the samples with a high percentage of L mucus (Figure 8).

Type III: this type presented a network aspect, with parallel and crossing fibres and pores of 1.5–7 μm in diameter. It was easily identifiable in the samples with a high percentage of S mucus (Figure 9).

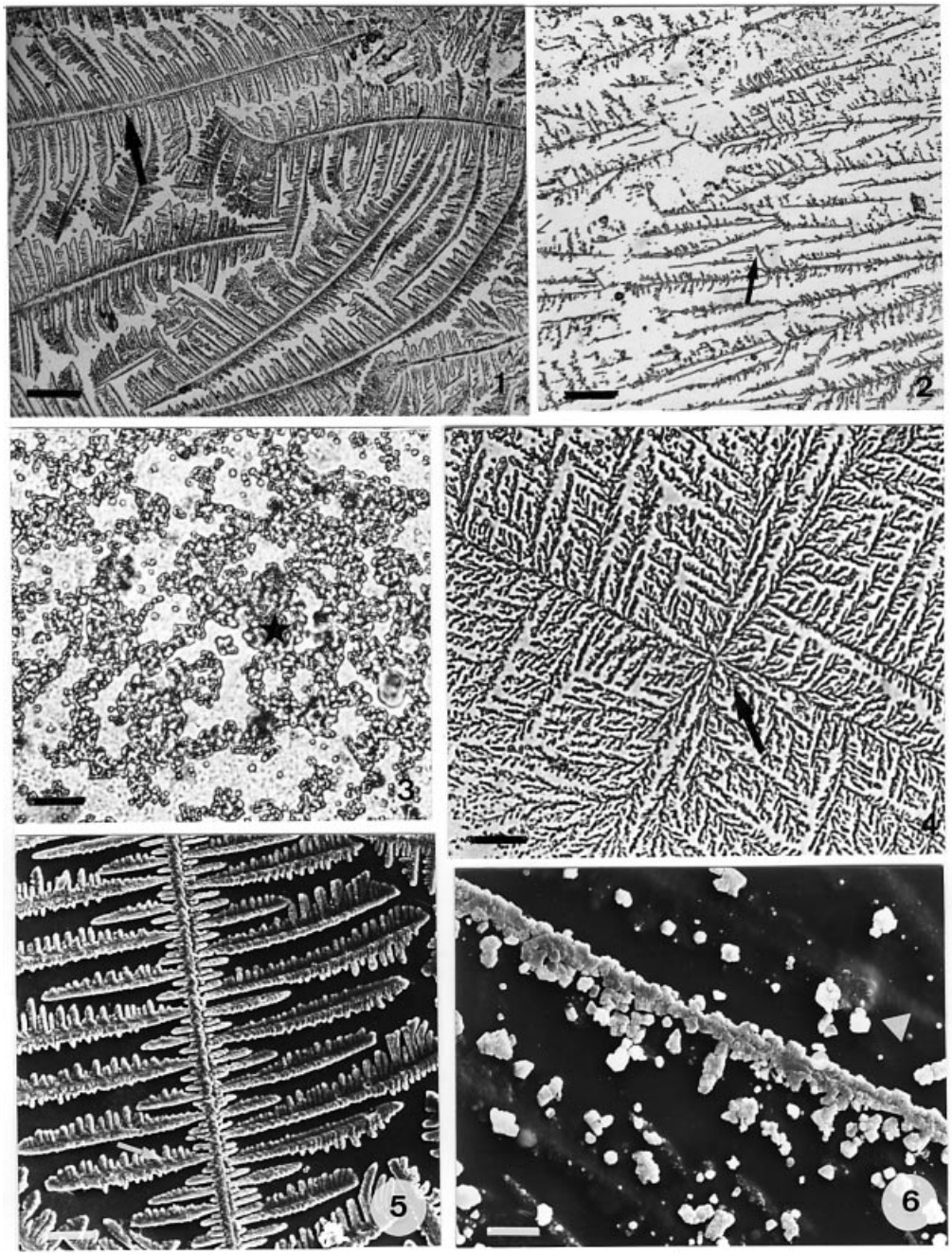

Figure 1. LM showing L mucus from the cervical canal in dried samples. It has a structure with a straight or curved central axis and branches protuding at a 90° angle (arrow) that can also act as an axis for new branches, again at a 90° angle (scale bar = 208 μm).

Figure 2. S mucus from the cervical canal in dried samples. In the S3 subtype we observe a parallel arrangement of crystals with short protruding branches (arrow) (scale bar = 89 μm).

Figure 3. G mucus from the cervical canal in dried samples. It has a high free-crystal (star) content with no predetermined form (scale bar = 31 μm).

Figure 4. P4 mucus from the cervical canal in dried samples. The ferning has two fundamental well-defined axes, with a 90° angle between them (arrow), and branches projecting from the main axis at a 60° angle (scale bar = 78 μm).

Figure 5. SEM showing L mucus from the cervical canal in dried samples. We can clearly observe two faces in most of the branches: one straight and the other dentate (arrow) (scale bar = 60 μm).

Figure 6. SEM showing S3 mucus from the cervical canal in dried samples. In the S3 subtype the crystals are joined and have branches. The organic substrate can be observed as shadows behind the crystals (triangle) (scale bar = 10.6 μm).

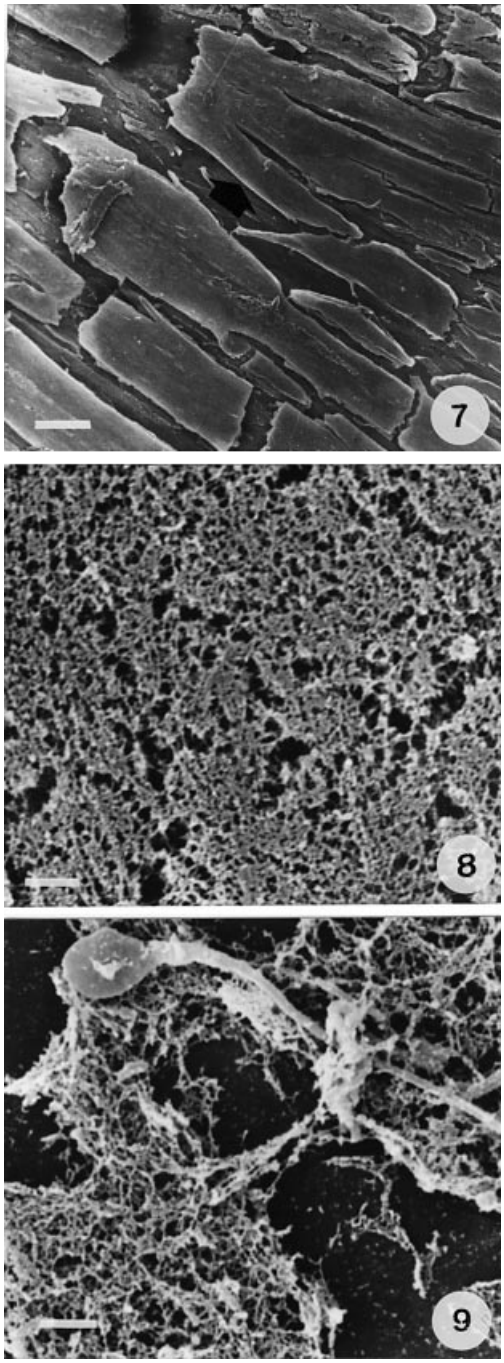

Figure 7. Type I mucus from the cervical canal in fixed samples. Compact mucus, with small pores is very common in samples with a high percentage of G mucus (60–94%). It frequently presents large plain surfaces (arrow), sometimes broken (scale bar = 16.6 μm).

Figure 8. Type II mucus from the cervical canal in fixed samples. It is not as compact as type I. The structure is similar to a marine sponge. It is common in the samples with a high percentage of L mucus (scale bar = 5.1 μm)

Figure 9. Type III mucus from the cervical canal in fixed samples. It presents a network aspect with crossing fibres and is easily identifiable in the samples with a high percentage of S mucus. A spermatozoon can be seen (scale bar = 4 μm).

Type IV: this type presented parallel folds and plain surfaces. The diameter of the pores measured 0.4–2 μm. This type could be identified in samples with at least 5% of P mucus.

Zones of the cervical crypts

LM: air-dried mucus samples

Zone of the L crypts: the mucus had the same ferning as the samples we had seen before from the middle of the cervix, although with certain differences. For example, the central axis was often curved and the branches were thinner, although they were at a 90° angle.

Zone of the S crypts: the morphology under light microscopy was similar to subtype S1. It consisted of a parallel arrangement of crystals which were close together but not joined (Figure 10).

Zone of the G crypts: the mucus was very similar to G mucus from the cervical opening. It consisted of free crystals and plenty of cells.

Zone of the P crypts: the mucus revealed mostly the P2 or Pa (Figure 11) subtypes.

SEM: air-dried mucus samples

Zone of the L crypts: L mucus in samples from the crypts presented the same morphology as the samples from the bulk but with a smaller ferning pattern (Figure 12). The size of the crystals was smaller (1.5–2 μm).

Zone of the S crypts: in the S mucus we observed very clearly the linear array of the crystals, which were not joined. Inside the crypts we saw only subtype S1 (Figure 13) .

Zone of the G crypts: in the G crypt samples we observed free crystals, alone or grouped, and without any defined form. The crystals were hexagonal, rounded or cubic.

Zone of the P crypts: in the samples from the P crypts we saw subtypes Pa and P2. P2 had a central axis with branches protruding at a 60° angle, similar to the samples from the canal. However, the most frequent subtype was Pa, the axes of which were not well defined.

SEM: fixed cervical mucus samples

Zone of the L crypts: a network structure was observed in the L cervical mucus. Most of this structure was similar to a marine sponge, but with different pores, ranging from 0.5 to 1.3 μm in diameter (Figure 14). The network was quite dense, although parallel or crossing fibres were sometimes found.

Zone of the S crypts: the S mucus from the crypts was less dense than the rest, so it was extended more easily than the rest of the different mucus types. It presented parallel or crossing fibres, with pores of a greater diameter (1.5–7 μm) than the L network (Figure 15).

Zone of the G crypts: the G mucus was difficult to spread out for analysis. It presented large plain surfaces and sometimes red blood cells or other cells. With a high magnification we were able to see the pores (0.1–0.5 μm in diameter).

Zone of the P crypts: in the P mucus type the morphology was different and new to us. We could see plain surfaces, tending to form parallel folds (Figure 16). Sometimes pores were observed on the surface (diameters of 0.4–2 μm).

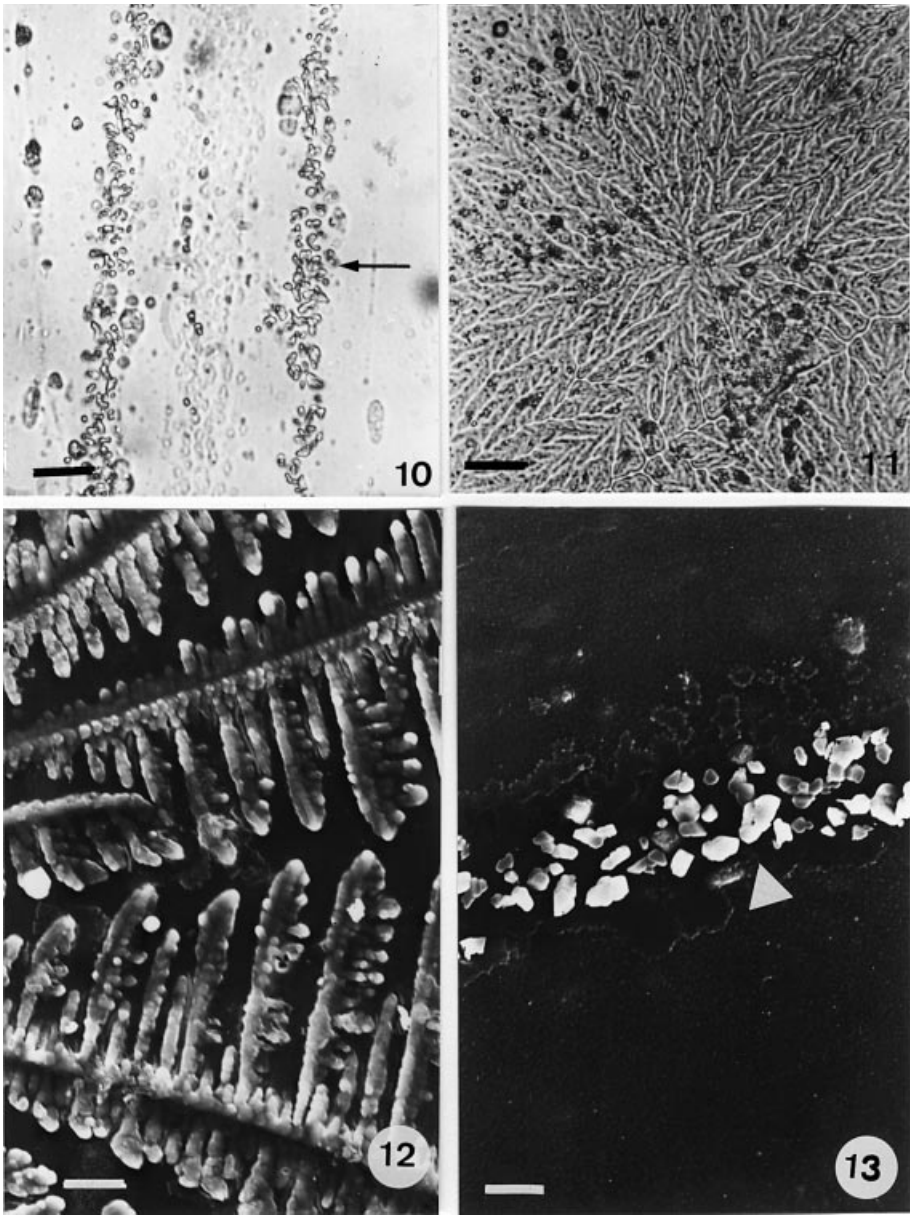

Figure 10. S mucus in dried samples from the crypts. The morphology with LM is similar to subtype S1, which consists of a parallel arrangement of crystals which are closer together but not joined (arrow). The crystals are large or rounded, and some cells can be observed (scale bar = 31 μm).

Figure 11. P mucus in dried samples from the crypts. Pa is the most frequent P subtype, the axes of which are not well defined (scale bar = 31 μm).

Figure 12. SEM of L mucus, in dried samples, from crypts presents the same morphology as the samples from the cervical canal, but with a smaller ferning. We can clearly observe the two faces of the branches, one straight and the other dentate (scale bar = 20 μm).

Figure 13. SEM of S mucus in dried samples from the crypts. We observe the linear array of the crystals, which are closer but not joined (triangle). The organic substrata behind the crystals can also be observed (scale bar = 10.5 μm).

Discussion

With LM we identified the four mucus types that were discovered and described by Odeblad (1997). Our results are successful and, similar to those obtained by Odeblad *et al*. (1983), with the 'spread-out' technique, in our laboratory proved to be: (i) quick; (ii) easy to perform at the different times of the cycle; and (iii) easy to evaluate, by counting the percentages of the different mucus types. It also allowed us to group the samples with the aid of the last menstruation date and complementary data. This can be very useful for improving the efficiency of the ferning test. Our observations confirmed that there are cyclic changes in the ferning of the mucus, depending on the day of the cycle that the samples are taken on, which can be expressed as percentages. This quality would allow it to be

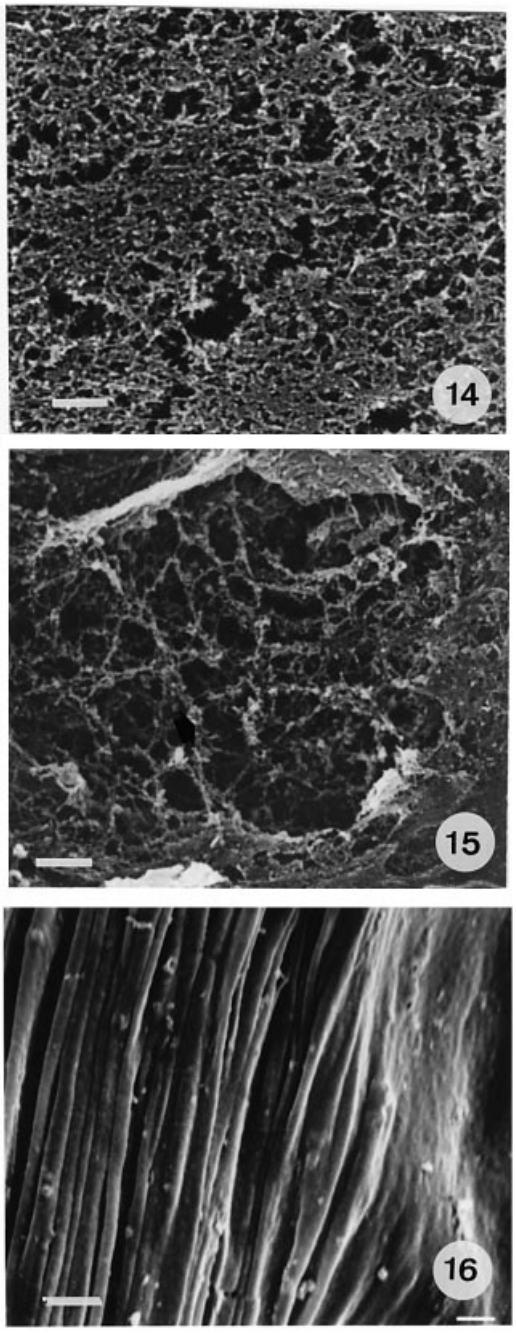

Figure 14. Fixed L mucus from the crypts. Most of the network structure observed in this mucus is similar to a marine sponge. The network is quite dense, although parallel or crossing fibres are sometimes found (scale bar = 2.5 μm).

Figure 15. S mucus in fixed samples from the crypts is less dense than the rest, so it extends more easily than any of the different mucus types. It presents parallel or crossing fibres (arrow) with pores of a larger diameter (scale bar = 7.1 μm).

Figure 16. P mucus from fixed sample from the crypts. It presents an interesting morphology, which consists of plain surfaces, tending to form parallel folds (scale bar = 1.4 μm).

used clinically, not only for problems of infertility but also for natural family planning.

One of the most important findings with regards to the biosynthesis of the cervical mucus is the location of different crypt zones inside the cervical canal that produce different mucus types. This finding was first suggested by Melén and Odeblad (1951) and subsequently supported by studies using NMR (Odeblad, 1966; 1997). The results obtained showed a very similar morphology between the crystallographic forms of the different types observed in 'spread-out' samples from the cervical canal, and in samples that came from the crypts and gave rise to interesting conclusions. First, it can be said that the samples from the different zones of the cervical crypts have different contents, with different morphological characteristics. Secondly, their crystallographic characteristics are very similar to those found in the samples from the cervical canal. Thirdly, our observations confirm the NMR studies that found different mucus types in specific crypts (Odeblad, 1966). Further biochemical and molecular biology studies are necessary to determine what factors are responsible for the differences between each mucus type.

A previous study on air-dried cervical mucus with SEM in samples taken throughout a single menstrual cycle of a proven fertile woman was reported by Zaneveld *et al*. (1975). The ferning was clearly observed by these authors during mid-cycle, as well as the free crystals in the luteal phase, but they did not distinguish the different mucus types.

In our study, we describe for the first time the four mucus types observed with SEM, namely G, L, P and S. This was made possible with the spread-out technique, which allowed us to separate the different mucus types. For example, the fact that S mucus tends to form a parallel arrangement may be related to Odeblad's idea regarding the filamentous nature of this mucus in normal conditions (Odeblad, 1997), which makes sperm migration through the cervix easier.

Several authors have shown interest in the morphological structure of cervical mucus, studying it with SEM in fixed mucus (Barros *et al*., 1985; Chrétien and Berthou, 1991; Vigil *et al*., 1991). The aim of these studies is to determine whether there is a relationship between the different phases of the woman's cycle and the cervical mucus structure, and they have concluded that during the ovarian cycle of the woman, cervical mucus undergoes changes in the pore diameters of the glycoprotein network, which could be associated with the estrogenic phases (increase in pore sizes) and luteal phases (decrease in pore sizes). The appearance of the network is considered tight in the gestagenic phase and loose with large pores and sometimes parallel fibres during the estrogenic phase (Chrétien *et al*., 1976). All these results are evaluated by the authors in accordance with Odeblad's 1968 model, where estrogenic and gestagenic mucus are described (Odeblad, 1968).

The main difference between our study and all the others is that we used the 'spread-out' technique, which allowed us to observe the four different mucus types very clearly. Furthermore, we did not consider the cervical mucus to be a homogeneous entity, but a mosaic of the four mucus types in various proportions during the cycle. Our results agree with the assumption that the cervical crypts producing each specific mucus type seem to be located in its specific area or zone of the cervix. In conclusion, the zones of cervical crypts are very specific areas of mucus synthesis where different mucus types

are produced, which will then combine to constitute what we know as cervical mucus.

Odeblad *et al*. (1983) characterized the different mucus types biophysically and crystallographically, but our study shows for the first time using SEM that they have a different ultrastructure. This strengthens the hypothesis about the heterogeneous entity of the cervical mucus, especially in the ovulatory and estrogenic phases of the menstrual cycle. If we try to explain Odeblad's model (Odeblad, 1997) using our own results, we can say that there are different cervical crypt zones secreting different mucus units with a different molecular architecture, which would combine to form what we know as cervical mucus and would probably have different functions. The four different morphological types (I, II, III and IV) correspond quite well with the four different mucus types described above in the samples from specific crypts. Type I corresponds to G mucus, type II to L mucus, type III to S mucus and type IV to P mucus. This information confirms mucus ultrastructure heterogeneity inside the cervical canal, due to the different units that make it up, and the changes that this mucus undergoes throughout the ovarian cycle. We can say that cervical mucus not only differs from the estrogenic to the luteal phase, but also that in the peri-ovulatory phase it is possible to find the co-existence of several types. It seems that secretions from specific crypts are mixed in different proportions in the cervical canal during the cycle. However, further studies on mucus biosynthesis are necessary, to confirm the biochemical differences between the mucus types.

In conclusion, human cervical mucus located inside the cervical canal is a morphological heterogeneous entity with different types of secretion, the proportions of which vary during the cycle. They show a different ferning and ultrastructure, related to the arrangement of the glycoprotein network, and are produced in different zones of the crypts in the cervix.

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