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Scanning electron and light microscopy study of the cervical mucus in women with polycystic ovary syndrome

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Abstract Two types of cervical mucus are recognized, oestrogenic and gestagenic. These are constituted by different subtypes, and their characteristics change depending on variations in the hormonal levels and on the existence of several pathologies. Our aim was to identify the ultrastructure and crystallization characteristics of the cervical mucus in women suffering from polycystic ovary syndrome, and to compare these characteristics with those of normal control women. Cervical mucus samples were taken from 10 women, 4 control group women (with normal ovulatory menstrual cycles) and 6 suffering from polycystic ovary syndrome (2 with ovulatory and 4 with anovulatory cycles). This mucus was characterized according to its ultrastructure and crystallization. The type of mucus obtained was related to the levels of oestradiol and progesterone present when the samples were taken. As regards mucus ultrastructure, differences were found between the control women and those with polycystic ovary syndrome and anovulatory menstrual cycles. Such variations were evident in the type of mesh and the average diameter of the mucus pores. Mucus crystallization in control women showed the usual oestrogenic disposition: fern-like (L, P2), rectilinear (S) or a hexagonal structure (P6). On the other hand, in women with polycystic ovary syndrome, indefinite mucus crystallizations were found, as well as crystallization patches resembling oestrogenic and gestagenic-like mucus. This study shows that the ultrastructure and crystallization characteristics of the cervical mucus in polycystic ovary syndrome women are different from those of control women. The latter would be dependent on their levels of oestradiol and progesterone.

Keywords cervical mucus, ultrastructure, polycystic ovary syndrome, network, mesh, crystallization

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Introduction

Human cervical mucus is a secretion produced by the cervical crypts, and shows variable rheological features. Mainly aqueous, its 90–95% water content increases to 98–99% during the periovulatory period [1]. This mucus has two phases, an aqueous phase containing inorganic ions, proteins, enzymes, immunoglobulins, amino acids and sugars [2], and a gel phase with high-molecular-mass components

[2]. During ovulation, the mucus has a lower viscosity [3] and is produced in a greater volume than over the rest of the cycle [3].

The cervical mucus has various important functions [3–9], two of which are worth mentioning here: (i) allowing for sperm transport, being the first barrier spermatozoa have to cross when ascending towards the fertilization site [5–7]; (ii) acting as a selective obstacle [7], permitting only some spermatozoa to migrate through the female genital tract and only during 6–7 days of the menstrual cycle [7–9]. Besides, mucus prevents the spermatozoon from undergoing a premature acrosome reaction – a modification that is necessary in order to go through the zonae pellucidae, penetrate the oocyte and fuse with it [10,11] – hence maintaining the fertile capacity of the spermatozoon [11,12]. Other functions of the mucus are to protect the female genital tract [6], to make spermatozoon survival possible [6,8,11] and to act as an antimicrobial barrier [13].

The high-molecular-mass components present in the gel phase of the cervical mucus are the mucins [2,3,14–24]. These are large ($M_r \sim 10^6$–10^7 Da) [15], highly glycosylated, viscoelastic glycoproteins [15] composed of 75% carbohydrates and 25% amino acids linked by means of *O*-glycosidic bonds between *N*-acetylgalactosamine and serine and threonin residues [15,16]. Mucins contain not only *N*-acetylgalactosamine, but also four other simple sugars: *N*-acetylglycosamine, fucose, galactose and sialic acids [17]. Mucins are encoded by *MUC* genes found in chromosomes 1, 3, 4, 7, 11, 12, and 19 [16]. More than 20 *MUC* genes have been identified in humans [16], and at least 13 mucins have been specifically found in the female genital tract; among the latter, gel-forming mucins are especially relevant: MUC2, MUC5AC, MUC5B and MUC6 [18–21]. Mucins would be responsible for the rheological properties of the mucus [22,23], controlling the structure of its gel phase [24].

The biophysical and biochemical features of the cervical mucus vary along the menstrual cycle [25–28], and the changes this mucus undergoes are highly related to serum steroid hormone levels and to ovulation [29–31]. Traditionally, and taking these changes into consideration, the cervical mucus has been classified as oestrogenic (E) or gestagenic (G) mucus [4,6,8,32,33]. However, based mainly on studies by Odeblad [4,26,32,34], and according to its crystallization pattern when air dried, it is known today that oestrogenic mucus is divided into three types: (i) type S, formed by small crystals arranged in parallel lines, supposedly the type responsible for spermatozoon transport and, in turn, subdivided into S1, S2 and S3 [6,8,14,26,34]; (ii) type L, with a fern-like morphology consisting of a central axis with 90° angle ramifications, probably offering support to type S mucus while preventing the ascent of abnormal spermatozoa [6,8,14,26,34]; and (iii) type P, similar in structure to type S, shows fern-like crystallization but with axis ramifications at 60° angles, and is divided into five subtypes (P6, Pa, P2, P4 and Pt) [6,8,14,26,34]. This classification is in agreement with scanning electron microscopy (SEM) studies that have found ultrastructural differences between samples of oestrogenic mucus [6,35–37], especially with regard to canalicular diameter, filament length and density of the mucus mesh. Considering G (gestagenic or progestative) mucus, this is secreted throughout the whole menstrual cycle, mainly in the early oestrogenic and during the luteal phase (or progesterone dependent phase), and its structure consists of a dense mesh that prevents the passing of spermatozoa [6,8,14,27,35]. On the other hand, when steroid hormone production decreases, as during amenorrhoea and post-partum lactating periods, small amounts of mucus resembling G mucus are secreted [38]; however, unlike the latter, post-partum mucus occasionally allows for spermatozoa migration [27,38].

Our aim was to identify, by means of SEM and light microscopy, the characteristics of the cervical mucus in polycystic ovary syndrome (PCOS) women (with both ovulatory and anovulatory menstrual cycles) and to compare them with the attributes present in the mucus of normal ovulatory menstrual cycle women, both regarding ultrastructure and crystallization patterns.

Methods

Women's approval

The present study protocol was approved by the Bioethics Committee of the Fundación Médica San Cristóbal, located in Vitacura, Santiago of Chile. Each woman gave written, informed consent to participate in the study prior to screening.

Women selection

Ten women attending Fundación Médica San Cristóbal regularly participated in the study. These women were divided as follows: (i) control women ($n = 4$), with normal ovulatory menstrual cycles; (ii) PCOS women with ovulatory menstrual cycles ($n = 2$) and (iii) PCOS women with anovulatory menstrual cycles ($n = 4$). In this study, PCOS diagnosis – an ovulatory dysfunction associated with hyperandrogenism [39,40] – was made according to the Rotterdam Consensus criteria [41,42]. These women were screened to rule out they did not suffer from other disorders such as non-classical congenital adrenal hyperplasia, thyroid dysfunction or hyperprolactinaemia, all of which could interfere with a conscientious diagnosis of PCOS [41,42]. Besides, none of the women in the study had undergone hormone treatment at least during the last year.

Hormonal measurements

The level of steroid hormones – oestradiol and progesterone – was determined by measurement of oestrone glucuronide (E1G) and pregnanediol glucuronide (PdG) metabolites in urine. The day of the cervical mucus screening, women were asked to supply an early morning urine sample. Subsequent urine samples were obtained every 5 days until their next menstruation. Specifically, E1G and PdG levels were measured using the Ovarian Monitor [43] to either verify or discard ovulation. It has been shown [44] that assessing urinary excretion of E1G and PdG by the use of the aforementioned method constitutes an accurate and trustworthy appraisal of the level of ovary hormones (oestradiol and progesterone) [44]. The evaluation of the levels of E1G and PdG by means of the Ovarian Monitor allowed us to identify the specific menstrual phase of each woman on screening and also to determine whether the menstrual cycle was ovulatory or anovulatory. In addition, during the mucus sampling, a transvaginal ultrasonography was carried out on each patient to assess the menstrual cycle stage by observing the follicular development on the ovaries [45], and the width and type of endometrium. All the ultrasonographic findings were in agreement with hormone values in determining the menstrual cycle stage.

Cervical mucus

Cervical mucus samples of each of the studied women were obtained through aspiration from the cervical canal lumen using an Aspirette^{TM} cannula.

Scanning electron microscopy

Characterization of the ultrastructure of the samples from both control and PCOS women was achieved through SEM according to the following procedure: each cervical mucus sample was fixed immediately after being obtained in a 3% glutaraldehyde (pentane-1,5-dial) solution in a 0.25 M cacodylate buffer at 4°C for 48 h to prevent morphological changes in the mucus mesh or in its hydratation and, therefore, alterations in the structures under observation. After the fixation period, the samples were washed twice in a 0.25 M cacodylate buffer for 30 min each, to remove the remaining fixer. Next, they were dehydrated in an ascending gradient in acetone (propanone) concentration. For this, the samples went through 30, 50, 70, 90, 100, 100 and 100% v/v concentration acetone solutions, remaining in each solution for 120 min. Final drying involved critical point dehydration in a Sorval centrifuge. Later, the mucus was removed from the cannula and mounted on slides to be shaded with palladium gold for 3 min at 18 mA, to allow processing with SEM as described in [9]. Finally, observations were made with a JEOL JSM-25SII scanning electron microscope.

Light microscopy

With the purpose of identifying the crystallization patterns of each woman's mucus, the latter was studied with light microscopy (LM) according to the following procedure: a small drop of the sample was spread out on a slide in all directions using a needle ('spread-out technique') [46]. Afterwards, the samples were air dried at room temperature for at least 15 min before the study. Next, the spread-out samples of mucus were studied with LM. To do so, 10 fields, chosen at random from the sample, were analysed using a 10 × 10 grid under the microscope eyepiece. The crystallization patterns of the mucus obtained from each woman were analysed on the basis of Odeblad's model [34].

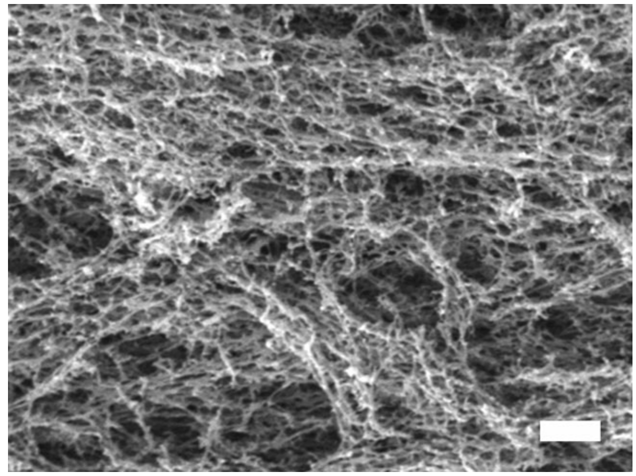

Fig. 1. Scanning electron microphotography showing the ultrastructure of the cervical mucus found in control women, with an average pore diameter of 15 μm. Bar = 20 μm.

Results

Hormonal levels

Based on the hormonal levels obtained for control women (ranges of 100–180 nmol/24 h and 2–4 μmol/24 h of E1G and PdG, respectively), the cervical mucus sample for each was obtained during the periovulatory phase of the menstrual cycle. At the same time, the hormonal levels of women with PCOS and ovulatory menstrual cycles (ranges of E1G and PdG of 135–140 nmol/24 h and 4.6–5.4 μmol/24 h, respectively) were similar to those of control women in the periovulatory phase. However, PCOS women with anovulatory menstrual cycles showed E1G levels ranging from 60 to 152 nmol/24 h and PdG levels in the range of 1–5 μmol/24 h.

Characterization of the ultrastructure of the cervical mucus

The ultrastructure of the cervical mucus in control women was found to present a lax appearance, with pores 15 μm in diameter [biggest part of pore ellipse, i.e. major axis (Fig. 1)]. On the other hand, the ultrastructure of the cervical mucus of PCOS women with ovulatory menstrual cycles showed similar characteristics to those of control women, with an average pore diameter of 8.4 μm (Fig. 2). Meanwhile, the ultrastructure of the cervical mucus in PCOS women with anovulatory menstrual cycles appeared to be denser and

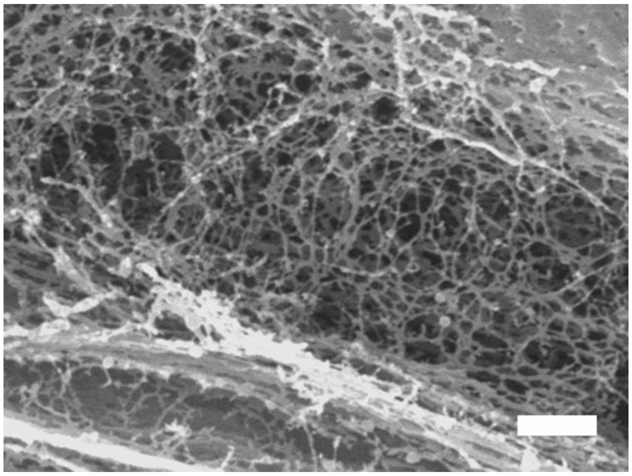

Fig. 2. Scanning electron microphotography showing the ultrastructure of the cervical mucus found in PCOS women with ovulatory menstrual cycles, with an average pore diameter of 8.4 μm. Bar = 20 μm.

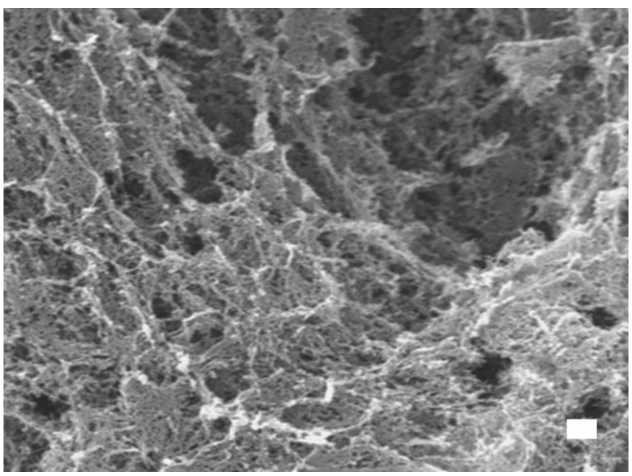

Fig. 3. Scanning electron microphotography showing the ultrastructure of the cervical mucus found in PCOS women with anovulatory menstrual cycles, with an average pore diameter of 1.8 μm. Bar = 5 μm.

more impenetrable than the others, with 1.8 μm diameter pores (Fig. 3).

Description of cervical mucus crystallization in control and PCOS women

The cervical mucus crystallizations of control women observed through LM presented a well-defined, identifiable pattern and a symmetric arrangement in agreement with the findings of other reports [6,8,14,36]; there was evidence of crystallization patterns of type L, subtype P2, subtype P3 and type S mucus (Fig. 4).

The cervical mucus of PCOS women with ovulatory menstrual cycles, observed through LM, showed crystallization (Fig. 5a) resembling type L mucus. On the other hand, the

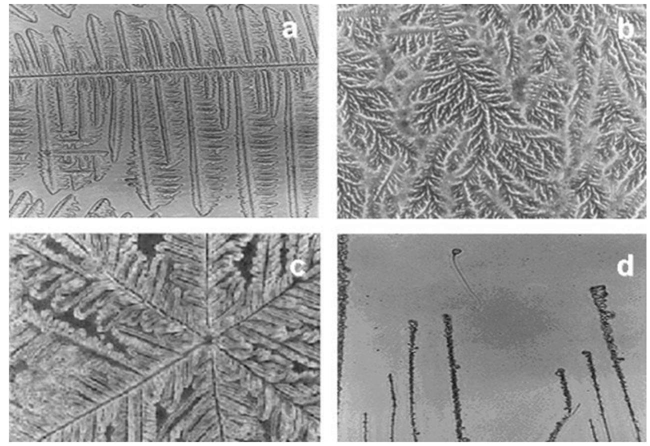

Fig. 4. Images obtained through light microscopy (400×) of the crystallization patterns of the cervical mucus in control women: (a) a crystallization pattern of type L mucus is observed; (b) crystallization pattern of P2 subtype mucus; (c) crystallization pattern of P6 subtype mucus, showing its typical star-like morphology with six well-defined axes and (d) crystallization pattern of type S mucus.

cervical mucus of PCOS women with anovulatory menstrual cycles showed a poorly defined and atypical crystallization (Fig. 5b); these women also presented crystallization (Fig. 5c) resembling type G mucus.

Discussion

The ultrastructure of the cervical mucus in women with PCOS and ovulatory menstrual cycles, observed through SEM, consisted, in general, of a mesh with a similar appearance to what has been described for the ultrastructure of the periovulatory cervical mucus [6,36,37]. The latter is in accordance with the values of sex steroids obtained by measuring hormonal levels since, according to these, the ultrastructure of the cervical mucus observed in PCOS women with ovulatory menstrual cycles should consist of a mesh of the cervical mucus with an ultrastructure similar to that of the cervical mucus found during the oestrogenic phase of the normal menstrual cycle, i.e. highly symmetric in appearance, with a recognizable mesh that is not too dense and shows well-defined pores [6,36,37]. SEM observations (Fig. 2) coincide with this description. It has been described [47] that the mesh of the oestrogenic cervical mucus should be characterized by having filaments that are interconnected, present a varied orientation and form canalicular units (or pores) [47], with a diameter in the range of 10–15 μm. In our study, however, the average pore diameter for PCOS women with ovulatory menstrual cycles was 8.4 μm (Fig. 2), indicating that in these women, the cervical mucus would be structurally related to the oestrogenic cervical mucus (Fig. 1) [37]. On the other hand, the ultrastructure of the cervical mucus of PCOS women with anovulatory menstrual cycles, as observed through SEM, consisted of a mesh that was much more compact, denser and impenetrable in appearance (Fig. 3) when compared to the

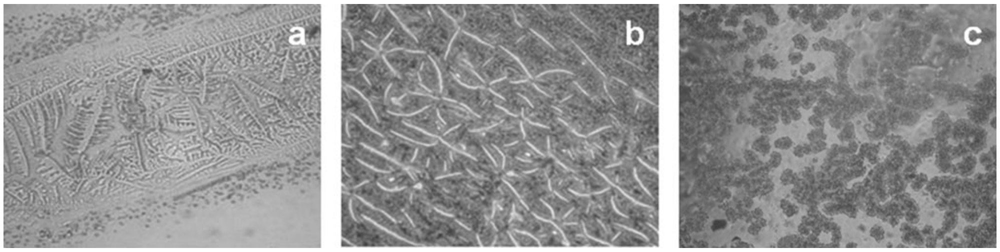

Fig. 5. Images obtained through light microscopy (200×) of cervical mucus crystallizations in PCOS women: (a) crystallization resembling type L mucus found in PCOS women with ovulatory menstrual cycles; (b) atypical mucus crystallization found in PCOS women with anovulatory menstrual cycles and (c) crystallization resembling type G mucus found in PCOS women with anovulatory menstrual cycles.

mesh observed in PCOS women with ovulatory menstrual cycles (Fig. 2) and, therefore, different from that reported [6,36,37] and found for women in the periovulatory period (Fig. 1). In addition, the average diameter of cervical mucus pores in PCOS women with anovulatory menstrual cycles (1.8 μm) is smaller than that reported [37] for oestrogenic cervical mucus (with pores ranging from 10 to 15 μm). This mucus shows a more compact and less symmetric appearance that would make it dense and impenetrable. These findings, together with LM crystallization (Fig. 5c), lead us to suggest that the cervical mucus in PCOS women with anovulatory menstrual cycles possesses an ultrastructural association with G mucus, since the latter shows a reduced average pore diameter [6,27]. In PCOS women with anovulatory menstrual cycles, the reduced average pore diameter of the cervical mucus could constitute a barrier against spermatozoa migration. Apparently, the reduced pore size and the denser quality of cervical mucus mesh present in PCOS women with anovulatory menstrual cycles as compared to those of control women could represent an additional cause that would explain, in part, the infertility disorders that some women suffering from this syndrome present as related to a deficient spermatozoa migration through the cervical mucus.

As regards crystallization patterns of the cervical mucus in control women, these showed a symmetric aspect (Fig. 4) and presented a ferning phenomenon or 'arborization', since fern-like crystallization (P2 subtype mucus) could be observed (Fig. 4b). A rectilinear arrangement was also found (type S mucus) (Fig. 4d), as well as a star-like arrangement with six well-defined axes that resemble hexagonally arranged 'feathers' (P6 subtype mucus) (Fig. 4c). The symmetric and well-defined appearance found for the cervical mucus crystallization in control women shows agreement with the different subtypes of crystallization of oestrogenic cervical mucus that have been reported [6,14,36].

Nevertheless, with reference to the cervical mucus of PCOS women, this showed different crystallizations (Fig. 5) than those of control women (Fig. 4), with a crystallization (Fig. 5b) that bears no resemblance to any of the crystallization patterns found for cervical mucus of control women in the ovulatory phase or to any of those previously reported [6,8,14,36]. However, zones (Fig. 5a and c) with similar characteristics to L- and G-type mucus crystallizations were also observed.

Considering the levels of sex steroids obtained in PCOS women with anovulatory menstrual cycles, E1G values found coincided with the follicular phase of a regular menstrual cycle; PdG levels, however, were higher than expected as compared to the basal levels present in a regular menstrual cycle during the same phase [44]. The latter indicates that the mentioned higher PdG levels (and, therefore, of progesterone) obtained could constitute one of the causes for alterations in the ultrastructure of the cervical mucus in PCOS women with anovulatory menstrual cycles, giving it the appearance of a denser and more compact mesh.

It is worth mentioning that women suffering from PCOS notice their cervical mucus to be sticky and find it less elastic and hydrated than the cervical mucus of the fertile period [39]. Among these women, alterations in the levels of sex steroid hormones would account for the stickiness of the cervical mucus as well as for the crystallization patterns found; in fact, it has been observed that, of all the physical properties of cervical mucus, crystallization is the most sensitive to changes at sex hormone levels [33]. It is important to consider that, in the cervical mucus of PCOS women, the changes in biophysical properties underlie variations in its characteristics (e.g. ultrastructure, crystallization); such changes in the biophysical features of the mucus would be caused by alterations in the levels of sex steroid hormones present in these women. This is in correspondence with other studies [20,25,28,33,48] in that the chemical composition and ultrastructure of the cervical mucus would depend on an interrelation between the levels of oestradiol and progesterone during the menstrual cycle.

In addition to PCOS, other pathologies have been described that may affect certain properties of the cervical mucus such as hydratation and the amount of mucus that is secreted. Among these disorders we can mention granulose cell tumours [49], bacterial infections of the female genital tract [26], psychological stress conditions [26,50], endocrine disorders in general [50,51] and female genital tract cancers [52–55], in addition to iatrogenic causes such as the intake of anti-oestrogens drugs [56] and oral contraceptives [57–59].

It would be relevant to consider here that the endocrine pathologies altering the properties of the cervical mucus probably act at the level of mucus glycoproteins, specifically at the level of mucins, since it has been observed that the variations in the endocrine pattern of the menstrual cycle lead to changes related to the differential presence of mucins in the cervical mucus [20].

Concluding remarks

On the basis of our findings, we suggest that there are differences in the characteristics of the mesh and in the pattern of crystallization of the cervical mucus between normal control women and PCOS women. To our knowledge, this would be the first contribution on this matter. These differences could be explained by the alterations in the levels of sex steroid hormones in PCOS women with anovulatory menstrual cycles; these differences would affect the characteristics of the cervical mucus in such women. It is also important to note that women with PCOS can be fertile and have ovulatory cycles, in which case cervical mucus characteristics are normal.

In general terms, it is the endocrine pattern typical of the menstrual cycle that controls the functions of the cervix and, therefore, also controls the biophysical properties of the cervical mucus [33]; subsequently, alterations in these endocrine patterns – especially as regards the levels of sex steroid hormones – will affect the characteristics of the cervical mucus.

Further studies should determine the glycoproteins present in the cervical mucus (particularly mucins) as differentially expressed both in control women and those suffering from PCOS, and others physiological and pathophysiological conditions.

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