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Cervical mucus: from biochemical structure to clinical implications

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1. ABSTRACT

Structure of human cervical mucus plays a pivotal role in female fertility and protection of reproductive health. Investigation of biochemical and biophysical structure of cervical mucus remains a challenge due to complex structural proteins, high content of oligosaccharides and cyclic variability of its structure. We present the current knowledge on chemical and biophysical features of cervical mucus and regulation of its secretion, relevant clinical observations and underexplored elements. The latter relates to biochemical background of physical properties and antimicrobial activity of cervical mucus, and regulation of its production.

2. INTRODUCTION

Cervical mucus is a glycoprotein gel produced by cervical glands (1-2). It plays a vital role in the protection of the uterine cavity from pathogens and controls survival and migration of sperm cells. At ovulation, estrogens increase hydration of mucus, which results in watery secretion with low viscoelasticity, allowing sperm cells to penetrate (3-4). During the luteal phase mucus is scanty, contains less water and provides an effective barrier to sperm cells.

Cyclic variability of cervical mucus has crucial, but not yet fully understood, impact on fertility and

Table 1. Summary of the mucus properties during different phases of the menstrual cycle. The source of each data is indicated by reference number

Phase of menstrual cycle	**ovulatory**	**preovulatory**	**luteal**
Estrogen levels	↑ high	↓ low	↑ high
Progesterone level	↓ low	↓ low	↑ high
Mucus amount	↑ high	↓ low	16, 21, 22
Mucus type	E (L, S, P)	G	3, 18, 19, 20
Sperm penetration and survival	↑ favorized	↓ prevented	3, 4, 17, 36, 37, 39
Protective capacity	↑ higher	↓ lower	45, 46
Viscoelasticity	↓ decreased	↑ increased	3, 4, 6,7,8, 26
Hydration	↑ high	↓ low	3 ,4, 24, 25, 69, 70
pH	↑ 8,0 (±)	↓ 6,2 (±)	55
Dried mucus appearance	anisotropic	isotropic crystals	20, 29, 30
Atomic forced microscopy image of mucus in aqueous environment	dispersed floating globules	dense filamentous (homogenous net of interconnected fibers)	34
MUC5B, MUC4 amount	↑ high	↓ low	23, 12
Mucin intermolecular aggregation	globular lateral	fibrous end-to-end	34
Glycosylation	more neutral oligosaccharides	more acidic oligosaccharides	24, 25
Cation content	↑ increased K+	↑ increased Ca2+	71
Sialidase activity	↑ increased	↓ decreased	60

reproductive health. Moreover, clinical significance of mucus in investigation of infertility or combating various infections needs further attention. Due to a very complex chemical and biophysical structure of cervical mucus, the available data are frequently incomplete and sometimes conflicting. This review is an attempt to collect relevant knowledge about the structure and production of cervical mucus and draws attention to still unrevealed mechanisms of cyclic hormonal regulation of mucus secretion.

3. STRUCTURE OF CERVICAL MUCUS

3.1. Biochemical structure

Biochemical structure of cervical mucus is complex and influenced by several variables such as endocrine status, which regulates the characteristic cyclic changes, and pH. Cervical mucus contains water (90-98 %) and a complex mixture of inorganic ions, amino acids, cholesterol, lipids, glucose, ascorbic acid, polysaccharides, mucins, plasma proteins, enzymes and bactericidal proteins (5). The viscoelastic gel properties of mucus depend on mucins, the major structural proteins in mucus, which are large glycosylated polymeric molecules linked together by disulfide bonds (6-8).

Twenty one distinct human mucin genes have been identified in reproductive, respiratory and gastrointestinal tract: MUCs 1-4, 5AC, 5B, 6-9, 11-13, 15-21 (9-11). Mucins are divided into three classes: secreted or gel-forming mucins, membrane-spanning mucins and small soluble mucins, but they all share a structural feature consisting of tandemly repeated amino acid sequences and a high content of O-glycosylation sites. The secreted mucins that form cervical mucus are MUC2, MUC5AC, MUC5B and MUC6 (12, 13). They have cysteine-rich domains that form disulfide bonds between monomers and in this way create multimers. Mucin glycoproteins have oligosaccharide chains that are, unlike the majority of glycoproteins, predominantly O-linked. The bound oligosaccharides highly increase the molecular weight of mucins, contributing to an increased solution volume and viscosity (9). The sugar groups contain sulphates and sialic acid residues that additionally consolidate the mucin structure and help in the protection from bacterial glycosidases and proteases (14-15).

3.2. Cyclic variability of the cervical mucus structure

Early reports demonstrated that amounts of mucus (as measured by wet weight) increased in the cervix at midcycle (16). Physical (rheologic) properties and biochemical structure of the cervical mucus also change during the menstrual cycle. Cyclical changes in the physicochemical properties are a consequence of hormonal changes (17). In 1968, the NMR studies revealed two types of cervical mucus, one with a high viscosity (G) and the other with a low viscosity (E) (18). The production of E mucus was considered to be stimulated by estrogens and G mucus by progesterone (18, 3). Subsequently, more mucus types were discovered, each of them correlated to different estrogens level (19, 20).

The maximum mucus production correlates closely with peak estrogen levels (21, 22). MUC5B and MUC4 are the major gel-forming and membrane-spanning mucin species in the endocervix, respectively (12). Expression analysis of mucin genes indicates that the amount of MUC5B varies during the menstrual cycle and peaks at ovulation (23). MUC4 and MUC5B are the predominant mucin messenger RNA (mRNA) transcripts present in the human endocervix during the menstrual cycle. The levels of both transcripts correlate inversely with serum progesterone level (12). The amount of MUC5B mucin displays a 3- to 7-fold increase at midcycle and drops dramatically in the luteal phase as mRNA levels drop and as blood progesterone levels increase (23).

Besides mucus quantity and production of mucin protein, cyclic variability includes changes in the water content, mucus glycosylation and pH (24, 25, Table 1). The altered biochemical structure contributes to the altered physical properties of mucus (26).

3.3. Structure of cervical mucus

Several approaches were used to study structure of cervical mucus. The simplest way was to dry cervical

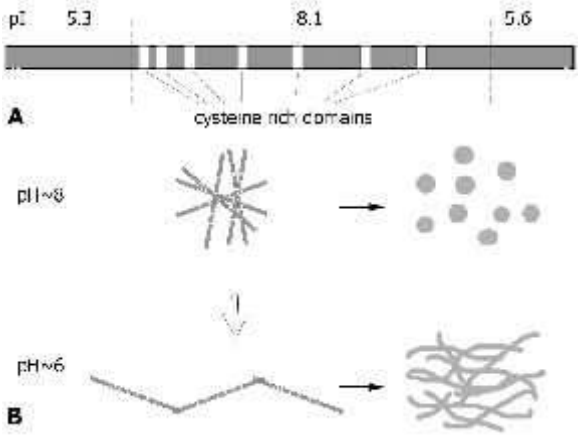

Figure 1. Diagram of the MUC5B primary structure and the proposed model of the mucin intermolecular aggregation at different pH. A. MUC5B consists of the three regions. The central region with low hydrophobicity and calculated isoelectric point 8.1 has cysteine rich domains with high hydrophobicity. The two outer regions show higher hydrophobicity and their calculated isoelectric points are 5.3 and 5.6, respectively. B. According to the proposed model of mucin intermolecular aggregation, at pH value compatible with the isoelectric point of the central region (pH~8), lateral intermolecular interactions contribute to the globular mucin structure visible by atomic forced microscopy (34). A pH shift from 8 to 6 causes switch from globular to fibrous mucus structure. In acidic environment (pH~6) compatible with the isoelectric points of the two outer regions, end-to-end intermolecular hydrophobic interactions form fibrous net.

mucus and observe it by a light microscope. Dried mucus has a typical fern-like appearance due to the presence of NaCl crystals (20, 27). The picture resembles dried saliva. Under the polarizing microscope, the fern-like structures appear as isotropic crystals (28). During ovulation, instead of the isotropic structures, anisotropic structures appear (29). Scanning electron microscopy revealed more accurate data about the mucus salt crystal structure (30). Different types of crystallization and different patterns of ultrastructure revealed different types of mucus secretions (20). The proportions of secretion types in mucus varied throughout the menstrual cycle. Moreover, different types of mucus secretions were isolated from different secretory zones of the crypts in the cervix, contributing to the hypothesis of a spatial distribution of different endocervical glands (31, 32).

Nuclear magnetic resonance and microscopy observations suggested filamentous mucus structure, where mucin glycoproteins were interconnected to form a network or bundles (3, 33).

Tapping mode atomic force microscopy (AFM) was used in an aqueous environment (not salt crystal structure) to image human cervical mucus during different phases of the menstrual cycle (34). Preovulatory mucus was arranged in a dense filamentous structure like a homogenous net of interconnected fibers. The average mesh size was of 500 ± 250 nm and the fibers' diameters ranged between 10 and 500 nm. The mesh size was too small for sperm cells to penetrate. Ovulatory mucus displays a rather different organization than preovulatory mucus, consisting of dispersed floating globules of aggregated mucin molecules (Figure 1). These ovulatory globules could explain decreased mucus viscosity and increased permeability to sperm. The image changes when the samples are dehydrated; the fern-like structure appears, same as previously reported for ovulatory mucus (20). This means that the globules that were imaged in hydrated and unfixed samples represent mucus structure more realistically.

A recent electron microscope study of the structure of the MUC5B mucin isolated by OptiPrep density gradient from saliva provided an insight into the structure of mucin in its unpacking state, as it is in the secretory granules. This allowed an approach to the question of how such large molecules were packed in secretory granules and how they might unpack into their linear polymeric form. This model of MUC5B organization demonstrated that MUC5B had a circular structure characterized by flexible carbohydrated chains connected around protein-rich nodes that are mainly composed of their NH2- and COOH- terminal protein domains (35).

4. BIOLOGY OF CERVICAL MUCUS – ITS CLINICAL ASPECTS

4.1. The role of cervical mucus in fertility

Cervical mucus is important for fertility, because it accepts, filters, prepares, stores and releases sperm for transport to the oviduct for fertilization (17). Sperm cells are incapable of survival and transport toward the ovum in the absence of sufficient levels of estrogenic-type mucus (17, 36, 37). Cervical mucus has a priming effect on sperm capacitation (38). It acts as a "passive filter" that selects and excludes the sperm cells with defective locomotive mechanisms (39). Migration through cervical mucus increases sperm ability to undergo acrosome reaction (40).

The capacity of mucus to accept sperm cells depends on the carbohydrate composition of the glycoproteins (41). Physical properties and sperm penetrability depend on the biochemical changes in cervical mucins, like changes in glycosylation or inter- and intramolecular bonds of mucins, as well as on environmental conditions, i.e. pH and HCO_3^- availability (26, 33, 42).

Bacterial mucinase activity may impede the mucus promotion of sperm progression by changing the biochemical properties of the mucus. Accordingly, it was demonstrated that the presence of Helicobacter pylori antibodies in the cervical mucus can be involved in female infertility (43).

4.2. Protective role of cervical mucus

Apart from its role in fertility, cervical mucus has a notable protective role. It provides a chemical and physical barrier to the infection, just as to the sperm cells.

Around the time of ovulation, due to the structural changes, it becomes permeable to sperm cells, but its protective capacities become more active and stronger. Ionic groups on oligosaccharides protect mucins from the action of bacterial glycosidases (14). Additionally, the sulphated mucins can inhibit adhesion of bacteria to target cells (44). Due to the presence of immunoglobulin-producing cells in the uterine cervix, the mucus contains secreted antibodies, which enhance protection against specific pathogens (27, 45). The antibacterial effect of mucus is confirmed by testing inhibition of bacterial growth on agar plates and is stronger after oral estrogen administration (45). The correlation of estrogen levels and protective capacity of cervical mucus is demonstrated in host-microbe interaction with Candida albicans. Cervical mucus with fucose-containing oligosaccharides reduces C. albicans binding to vaginal epithelial cells (46). Expression of mouse Fut2 gene encoding an α-1,2-fucosyltransferase that elaborates α-1,2-fucose mucin residues is most prominent in the glandular epithelium of the endocervix during estrus and is stimulated by estradiol treatment of ovariectomized mice (47).

4.3. Cervical mucus as a sign of ovulation

Mucus is also an important element for estimating the time of ovulation (22, 48 - 50). It has been used by women from many nations and cultures to recognize the fertile phase of menstrual cycle for the purpose of fertility awareness (51). Fertility awareness allows understanding and making informed decisions about reproductive and sexual health. It can help couples to plan pregnancies as well as to avoid them. Being familiar with their own mucus pattern helps women to detect gynecological disorders and to consult a physician (52). Since such gynecological disorders may represent the cause of infertility, monitoring mucus characteristics can be useful in detection and therapy of the cause of infertility (53).

5. BIOCHEMICAL BACKGROUND OF MUCUS PHYSICAL PROPERTIES

Mucins are responsible for viscoelastic gel properties of mucus. Although during ovulation high mucin content should increase mucus viscosity, the viscosity actually decreases. Factors determining the periovulatory mucus lower viscosity, and therefore increased permeability to sperm, are still a matter of consideration. The initial theory of increased water content is a partial explanation (25). Subsequent data about biochemical structure, pH and enzymatic activity of cervical mucus enabled further hypotheses to be established.

5.1. pH impact to mucin structure

Mucus pH changes before ovulation and returns to the previous level after ovulation (54). It is an important determinant of sperm-mucus interaction (55). Influence of pH on the structure of the newly evidenced mucin globules was investigated by lowering the pH of the ovulatory mucus sample to 6. The result was a dramatic modification of mucin structure with the disappearance of globules and appearance of a network that closely resembles the preovulatory mucus (34).

The phenomenon is explained by the primary structure of MUC5B (Figure 1). It has three well-resolved regions: a central, highly deterministic region between the residues 1100 and 3700 with lower hydrophobicity values, and two outer regions of higher hydrophobicity. The central region is regularly spaced by conspicuous hydrophobicity peaks exactly matching low determinism sequences that can be identified as the cysteine rich domains. Calculated isoelectric point of the central region is 8.1, and its value in the left and right outer region is 5.3 and 5.6, respectively. The central region with higher isoelectric point has a low hydrophobicity and high determinism, i.e. a high propensity to aggregation, folding and flexibility (56). Its cysteine-rich domains stabilize aggregates by forming intermolecular disulfur bondings. The described features of the central region favor globular mucin structure at the pH value compatible with the isoelectric point of the central region. This pH is close to the pH of the ovulatory mucus. By simple acidification to the pH value compatible with the isoelectric point of the two outer domains, the globular mucus structure switches to fibrous. In acidic environments the two outer hydrophobic domains can form end-to-end intermolecular hydrophobic interaction that forms the fibrous net. The electrostatic repulsion in the central region hinders lateral intermolecular aggregation (34).

A similar pH-induced conformational change was described in the case of pig gastric mucin. This conformational change results in a sol-gel phase transition of mucin at higher concentrations and prevents the stomach from being digested by its own secretion (57).

Ovulatory mucus with low viscosity and high sperm permeability can be transformed to the highly viscous mucus with no sperm permeability by simple acidification. This finding minimizes the relevance of compositional changes, but still does not nullify it.

5.2. Mucin glycosylation

Mucus varies in carbohydrate composition and structure during menstrual cycle, particularly in the sugar residues containing sulfate groups and sialic acid residues (6, 14, 24). The charged molecules on sugars consolidate and protect the mucin structure, but their exact role in mucus rheologic properties remains elusive. It is supposed that the terminal glycosylation of mucins may be the main determining factor of the rheological properties of mucus (58). It is hypothesized that the mutual repulsive charge both between the mucin molecules and between the carbohydrate side chains of individual mucins is lost, the arrangement of mucin molecules in the solution is altered and the viscosity of the mucus gel decreases.

The complete proteome and mucin analysis of the human cervical mucus during the menstrual cycle indicated that the most remarkable difference was a shift in the mucin glycosylation. The O-glycans were virtually identical in the mucins before and after ovulation but were substantially different at ovulation. During ovulation there were relatively more neutral oligosaccharides than acidic ones

(sulphated and sialidated) (26). Although the new data about the mucus composition variability were revealed, the physiological importance of this shift and its correlation to rheologic status of mucus remained unexplained.

The shift from neutral to acidic oligosaccharides corresponds to the mucus pH shift from 8 to 6, and the change in the viscosity (Table 1). Does the pH shift cause the glycosylation shift, or the change in oligosaccharide content influences the pH, or both are interindependent? What is the exact role of pH in secretion and maturation of cervical mucus?

5.3. Enzyme activities

Enzymes involved in mucin breakdown are proteases, sialidases, other glycosidases and sulphatases. Their activities change mucin structure and hence its physical properties. These enzymes may influence the physical barrier to sperm cells and pathogens or may enhance bacterial adhesion and hence colonization (58). Proteases can lead to reduced viscoelasticity and disruption of gel structure by an initial cleavage at non-glycosylated regions. The initial HCO_3^- triggered mucin decondensation enables enzymes to access and cleave stabilizing intermolecular bonds of the NH_2^- and $COOH^-$terminal protein domains in the nodes of the globular MUC5B, and bring the mucin to its linear conformation (35, 59).

Since sulphated and sialidated glycans influence the hydration and chemical and physical properties of mucus (15), enzymes that change the oligosaccharide content of mucus could also change its physical properties.

A study of sialidase activity in genital tract secretions revealed that sialidase activity in cervical mucus of healthy women reached a maximum in the ovulatory phase (60). An increase in sialidase activity could play a role in modifying the rheologic and ultrastructural properties of cervical mucus.

6. MUCUS SECRETION

6.1. Secretory epithelium

Endocervical glands are duct-lacking invaginations within the cervical stroma with openings to the cervical canal. The lumen of the cervix and the endocervical glands is lined by a columnar epithelium that secretes cervical mucus. Ultrastructural analysis of endocervical glands defined three cell types in the epithelium: subcolumnar basal cells, mucus-secreting cells and ciliated columnar cells (61). The cilliated cells contribute to mucus production by ion and water exchange activity, which modulates the hydration of mucin molecules (62). Cytochemical studies with peroxidatic activity revealed two cell types of glandular cells, but their functional difference was not explained (63).

Histochemical, immunohistochemical and molecular studies demonstrated that endocervical epithelium undergoes cyclic, estrogen or progesterone dependent changes (12, 24, 47). Additionally, there is a hypothesis that endocervical glands with different spatial distribution produce different mucus types depending on estrogen stimulation (31, 32, 20). According to this hypothesis, the glands located in the upper portion of the cervical canal would secrete less viscous mucus, and the glands from the lower parts would produce more viscous mucus.

6.2. Mechanism of mucus secretion

Biosynthesis and assembly of secreted, gel-forming mucins takes place in the endoplasmic reticulum and Golgi complex. Fully processed, folded, glycosylated, sulfated and polymerized gel-forming mucins are stored in high amounts in large secretory granules that occupy the majority of the cytoplasm in mucous cells (9). Vesicles filled with mucus were also found outside the mucus cells and were called exosome-like vesicles (60, 64, 65). Their role is not yet fully understood. Although it is known that mucins may be secreted constitutively, by exocytosis of granules or small vesicles, or via a regulated pathway by exocytosis of granules, an exact mechanism how the mucins are organized in the secretory cells and how they are released is unclear. As a process of exocytosis commences, the condensed mucin macromolecules in secretory granules undergo abrupt swelling (66). The theory that explains the swelling mechanism by increased intragranular water content and diffusal motion of mucin molecules is now replaced by the theory of charge repulsion of the mucin polyionic residues (60).

Changes in pH and calcium concentration affect mucin granules swelling (64). Hence, regulation of Ca^{2+} and H^+ concentrations in the cervical canal can influence mucin hydration and viscoelastic properties of mucus. Indeed, the dominant ion in the secretory granules is calcium. Polyanyonic mucins are highly condensed within granules as a result of high concentrations of Ca^{2+} and H^+ that shield negatively charged sites on mucins from electrostatic repulsion (62). Once the secretory pore is formed, Na^+ ions enter the mucin granule, and start Ca^{2+} ion replacement. Since, according to electroneutrality principle, two Na^+ are required for each Ca^{2+}, the increased counterions inside the gel raise the osmotic pressure, water molecules move into the gel and the granules swell (62). The process of removing the shielding cations from mucins is more efficient if the cations are previously attracted by HCO_3^- (42). The initial expansion allows enzymes to cleave intramolecular covalent crosslinks and to complete the process of mucin unpacking (35). Additionally, HCO_3^- ions are crucial for normal release of gel-forming mucins to form transportable mucus. In the absence of HCO_3^-, mucus remains stuck within the crypts of the cervico-uterine epithelium, i.e. within the lumens of the mucus glands. Poor HCO_3^- secretion seems to be a component of low and reduced fertility as in cases of women with cystic fibrosis (67). $NaHCO_3$ vaginal douching reduces the viscoelasticity of cervical mucus and improves the sperm penetration test and post coital test (68).

6.3. Regulation of mucus secretion – role of cyclic hormones

There are many indicators of correlation between mucus production and cyclic hormones level. Expression of

MUC genes is shown to be in correlation to progesterone levels (12). Also, the flow of water towards the cervical lumen (69, 70), increased K^+ and decreased Ca^{2+} in the cervical canal (71), pH change (72) and antibacterial effect of mucus (45) follow estrogen level rise. However, direct evidence and a mechanism of estrogen or progesteron regulation of mucus production is not yet provided.

Cyclic hormone regulation of mucus production is not a simple and straightforward action, since mucus production comprises diverse intracellular and extracellular events. Thus, estrogen effect on mucus abundance and viscosity should be a cumulative result of estrogen dependent changes in mucin gene expression, water flow, ion exchange, pH, HCO_3^- supply, glycosylation, mucinase activity and velocity of mucus secretion and decondensation.

The events taking part in mucus production could be divided into "slow" and "fast" events. Slow events are expression of mucin genes, mucin protein synthesis and glycosylation, surrounding connective tissue swelling, etc. MUC5B protein level reaches its peak at least one day after the mRNA levels peak (23). On the other hand, mucin granules swelling and mucin expansion occurs within seconds (60). If both events are regulated by estrogen, then two distinct mechanisms of estrogen regulation should take place.

Estrogen receptors (ER), like other steroid receptors, are largely located in the cytosol. Hormone binding to the receptors triggers their migration into the nucleus, dimerization, and binding to hormone-response elements on DNA. As a consequence, gene transcription is initiated like that of *Muc5b*. On the other hand, some estrogen receptors may associate with cell membranes and induce rapid, nongenomic activation of downstream signaling pathways (73). A candidate estrogen receptor that would mediate such nongenomic estrogen effect was GPR30, an intracellular transmembrane estrogen receptor (74). Later, GPR30 was confirmed not to be an ER, but that it could just facilitate membrane-initiated steroid signaling under limited circumstances (75). Nevertheless, estrogen receptors that usually reside in the cytosol, can also be located at the cellular membrane and form complexes with G proteins, striatin, receptor and non-receptor tyrosine kinases (75, 76). Estrogen receptor α is present in the human endocervix tissue (77, our unpublished data). Binding estrogen receptor α to striatin leads to rapid estrogen-induced increase of Ca^{2+} and nitric oxide levels (78). Such non-genomic estrogen activity could be involved in regulation of the "fast" events in mucus production, especially those that regulate intracellular and extracellular ion concentrations and pH.

7. PERSPECTIVES

The final intention of the mucus research is to find out therapeutic approaches to treat and prevent infertility and infections of reproductive tract. Thus, two key questions that should be answered are: "What mucus features are required for its fertility and protection role?" and "How the mucus achieves those features?".

The present knowledge of the cervical mucus structure and function is a mosaic that only partially answers the questions. There is a consensus that biophysical properties of the mucus are crucial for sperm penetrability and hence for the fertility. The conditions that have to be fulfilled to achieve these properties are not yet defined: Brunelli *et al.* emphasize the role of pH, while other authors give priority to ion concentrations or water flow (34, 42). Biochemical content of the mucus seems to have minor contribution to fertility, but has an impact to protective properties of the mucus (26, 46).

The investigations of the chemical and biophysical properties of the mucus *in vitro* are very helpful for understanding of its function. Direct application of these understandings to control the viscoelastic properties of mucus could be a useful fertility strategy, but its applicability is questionable (68). Research of the mucus secretion and maturation in endocervical glands can give more valuable data about the inner and outer factors that contribute to and regulate mucus production. Those factors may be the targets of a possible therapy.

The cellular regulation of mucus secretion and variability is still almost unexplored field. This review just indicated a few elements of this regulation, but it does not comprise all subjects that could be involved in the regulation of endocervical epithelial activity. For example, investigation of neurogenic activation or interaction between secretory and cilliary cells could give a new insight into mucus production regulation. Altogether, more thorough studies with clinical data are required to understand cervical mucus and to utilize this understanding to improve therapeutic approaches in reproductive diseases and infertility treatment.

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