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An Observation of the Microstructure of Cervical Mucus in Cowsduring the Proestrus, Estrus, and Metestrus Stages and the Impact on Sperm Penetration Ability

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Abstract: In addition to providing energy, cervical mucus also acts as a barrier to prevent sperm from reaching the egg. The purpose of this research is to examine the microstructure of dairy cow cervical mucus and how it influences sperm permeability during the four menstrual cycles. As part of the experiment, cervical mucus was collected from 60 Holstein cows at various points in time. After the mucus crystallized, its various forms were noted, and its proportions were studied. Tests for sperm permeability were conducted using cervical mucus from various estrous phases and the number of sperm that passed through them. Scanning electron microscopy was then utilized to examine the mucus's ultrastructure and quantify the micro-pore diameters. According to the findings, there are four distinct kinds of cervical mucus produced by cows during their estrous cycles: L, S, P, and G. The relative proportions of these types vary. On one hand, the L type was much more common than the other kinds during the proestrus ($p < 0.05$); on the other hand, the S type was much more common than the other types during estrus ($p < 0.05$); and finally, on the third hand, the p type was much more common than the other types during metestrus ($p < 0.05$). There were no statistically significant changes in the micro-pore diameters of the same cervical mucus type throughout the several estrous stages ($p > 0.05$). Nevertheless, the four kinds showed significant variations in micro-pore diameters during the same estrous period ($p < 0.05$). Permeation of the cervical mucus by sperm was substantially greater during estrus and metestrus compared to proestrus ($p < 0.05$). Research on dairy cow cervical mucus has data support from this study.

Keywords: topics covered include: sperm penetration tests, cervical mucous, the estrous cycle, scanning electron microscopy, and dairy cows.

Introduction

Cervical mucus is a constantly changing fluid mainly composed of water, lipids, choles- terol, carbohydrates, inorganic ions, proteins, and other components, with a microstructure resembling a “mesh” [1]. The size of the “mesh” is regulated by the changes in estrogen and progesterone around the time of ovulation, to facilitate sperm passage through the reproductive tract during the optimal period [2,3]. The hydrodynamic properties of cervi-cal mucus impede sperm passage through the cervix during the luteal phase, when it is present in minimal quantities, characterized by high viscosity and opacity [4,5]. However,at ovulation, the production of cervical mucus increases, transforming it into a highly hydrated liquid, with the maximum permeability for sperm at this stage [6,7]. In human research, observations of cervical mucus can be used to diagnose certain reproductive tract diseases and to determine the precise phase of an individual’s physiological cycle [8–10].In dairy farming, cows also exude cervical mucus during estrus, but there is relatively little research on cervical mucus in cows. The microstructure of cervical mucus and its efficiency in allowing sperm passage during different phases of estrus in cows are worthy of study. The estrous cycle of dairy cows is primarily divided into four phases: the proestrusphase, the estrus phase, the metestrus phase, and the diestrus phase [11]. Cows exhibit mounting behavior during estrus, with ovulation typically occurring within 10–15 h afterthe initiation of mounting behavior [12]. Accurately determining the timing of ovulation in dairy cows and conducting mating or artificial insemination at the exact time can effectively improve the fertilization rate of dairy cows.

The crystalline pattern (dendritic) of cervical mucus can be utilized to detect estrus [13,14]. Cervical mucus is primarily classified into four types: Type L, Type S, Type P, and Type G, these four types of cervical mucus do not exist in isolation but are mixed in different combi- nations during different periods

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[15,16]. The different levels of estrogen and progesterone at various stages result in changes in the amount of each type, thereby causing the cervical mucus to differ in its final composition [2]. Type L cervical mucus begins to be secreted 5 days before ovulation, with a microstructure pore size ranging from 0.4 to 3 μm [15,16]. Since the diameter of the sperm head is between 4 and 7 μm [17], cervical mucus of the L-type can, to some extent, inhibit the entry of sperm [15,16]. The crystalline state of Type L mucus presents a shape with a straight or curved central axis, with branches extendingat a 90° angle; Type S cervical mucus is secreted around the time of ovulation, with a microstructure pore size ranging from 1.5 to 7 μm, forming a vast "highway" through which sperm can swim smoothly once they come into contact with Type S cervical mucus. The crystalline state of Type S mucus features parallel arranged crystals with short and prominent branches; Type P cervical mucus is secreted at the end of the cervix, with a microstructure pore size between that of Type L and Type S, ranging from 0.4 to 4 μm, serving a filtering and energy-providing function for sperm [15,16]. The crystalline stateof Type P mucus presents two basic axes with a 90° angle between them; Type G cervical mucus often appears at various stages of infertility and can form a mucus plug in the cervix, thereby sealing the cervix and preventing sperm from entering the uterus [15,16]. Type G cervical mucus also functions to protect the reproductive tract from external contamina-tion [15,16]. The majority of Type G cervical mucus is coral-like in appearance, without a fixed shape [15,16]. Whether the same pattern of cervical mucus changes exists in dairy cows is worthy of study.

Cervical mucus has different microstructures at different times, which can act as a special barrier for sperm, efficiently filtering sperm [18]. This experiment selected cervical mucus from dairy cows at different time points during estrus to observe the microstructure of the cervical mucus and conduct sperm penetration experiments. The results indicate that the microstructure of cervical mucus from dairy cows during different phases of estrus shows regular changes, and the number of penetrating sperm varies with the changes inthe estrous cycle. This study investigates the microstructure of cervical mucus and sperm penetration ability at different time points during the estrous period in dairy cows, provid- ing support for the identification of ovulation, and the timing of artificial insemination indairy cows.

Materials and Methods

Ethics Statement

All animal experiments were conducted in accordance with the "Regulations and Guidelines for the Management of Experimental Animals" established by the Ministryof Science and Technology (Beijing, China, 2020 revision). This study was approved by the Institutional Animal Care and Use Committee of Tarim University, Xinjiang, China (protocol code DWBH20220101; approval date: 1 January 2022).

Materials

Cervical Mucus: A total of 60 Holstein cows aged 3–5 years with similar body condi- tions and reared in the same environment were selected. The cows selected are multiparous dairy cows that have previously undergone artificial insemination. During the trial period, these cows were all in a non-pregnant state. Cervical mucus samples were collected on the day before estrus (proestrus), the day of estrus (estrus), and the day after estrus (metestrus)from each cow (all cows exhibited natural estrus). The collection of cervical mucus was performed at 24 h intervals, as illustrated in Figure 1 (samples were collected on day 21 of the previous estrous cycle, and on day 1 and day 2 of the current estrous cycle).

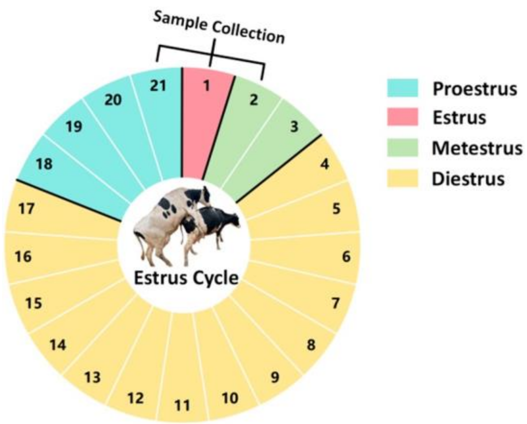

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Figure 1. Diagram of the estrous cycle in dairy cows.

Semen: Commercially available conventional frozen-thawed semen was used. Com-mercially available frozen semen is stored in liquid nitrogen. When thawing semen, remove the frozen semen from liquid nitrogen and quickly place it into a 38 °C water bath. Gentlyshake it for 30 s, then test the vitality and density, and utilize semen with a vitality rate exceeding 0.8 (the sperm concentration of the semen in the cryostraw is 150 million/mL).

Main Reagents and Instruments: A total of 2.5% glutaraldehyde, acetone, optical microscope, critical point drying apparatus, scanning electron microscope, sperm counting chamber, glass slides, and semen straws (0.25 mL per straw).

Collection of Cervical Mucus

The collection of cervical mucus from dairy cows during different phases of estrus was performed by gently pressing the cervix and vagina through the rectum. The cows were first restrained, and the feces within the rectum were cleared. Subsequently, the anus and vulva were cleaned. The collector gently pressed the cervix and vagina through the rectum, repeatedly applying pressure from the cervix towards the vaginal opening until the cervical mucus flowed out. The mucus was then collected using a clean beaker. If cows with uterine inflammation were found during sample collection, they were immediatelydiscarded, and only samples with clear and transparent cervical mucus were collected.

Observation of Cervical Mucus Crystallization

The observation of cervical mucus crystallization in dairy cows during different estrousphases involved initially pipetting 20 μL of cervical mucus onto a glass slide and spreadingit evenly. The slide was then air-dried at room temperature for 30 min to ensure that the cervical mucus on the slide was in a dried crystalline state. An optical microscope was used to observe the air-dried cervical mucus. The crystallization patterns of cervicalmucus during different estrous phases in cows were analyzed using the model proposedby Mikaela Menarguez [16]. Statistical analysis was performed on the quantity of each type of cervical mucus. For each sample, the five fields of view were randomly selected for observation, and each sample was repeated at least three times. This study involved thesimultaneous observation by 5 individuals and their evaluation of the samples.

Scanning Electron Microscopy Was Used to Observe the Microstructure of Cervical Mucus inCows at Different Stages of Estrus

Cervical mucus samples from different estrous phases were immediately fixed in 2.5% glutaraldehyde solution at 4 °C for 48 h after collection. Following fixation, the samples were washed twice in 0.25 M cacodylate buffer for 30 min each to remove the glutaralde-hyde. Subsequently, the samples were dehydrated through a series of concentrations of acetone (30, 50, 70, 90, 100% *v*/*v*), with each concentration of acetone used for 2 h of dehydration. The samples were then dried using a Leica EM CPD300 automatic critical point dryer (Leica EM CPD300, Leica, Germany), metallized with a Cressington 108Auto high-performance automated sputter coater (Cressington 108Auto, Cressington, UK), and finally observed with a Thermo Scientific Apreo S scanning electron microscope (Apreo, Thermo Fisher Scientific, USA). Statistical analysis was conducted on the micro-pore sizes of the cervical mucus, with each sample repeated at least three times. This study involved the simultaneous observation by 5 individuals and their evaluation of the samples.

Study on the Sperm Penetration Ability of Cervical Mucus in Dairy Cows during DifferentEstrous Phases

The sperm penetration experiments were refined based on the work of Tang, S et al [19].Sperm penetration experiments were conducted using empty semen straws (as shown in Figure 2); with an available length of 10 cm for each straw, the capacity is 200 μL. To facilitate measurement, a mark was made at 1 cm from the opening of the semen straw using a marker pen. Cervical mucus was first aspirated and filled into the straw up to the 1 cm mark from the opening (180 μL cervical mucus). Then, semen was added with a motility rate exceeding 0.8 into each straw, adding approximately 20 μL of semen per frozen semen straw at a concentration of 150 million/mL. The filled semen straw was placed horizontally in a 37 °C, 5% CO_2 incubator for 30 min. Subsequently, the straw was cut directly at the midpoint, and the mucus from the end, along with the penetrating sperm, was transferred into a 1.5 mL centrifuge tube. This was then centrifuged at a speed of 300 g/min for a duration of 5 min; the supernatant was subsequently discarded, and the remaining 20 μL of liquid was gently homogenized through pipetting. Finally, spermcounting was conducted

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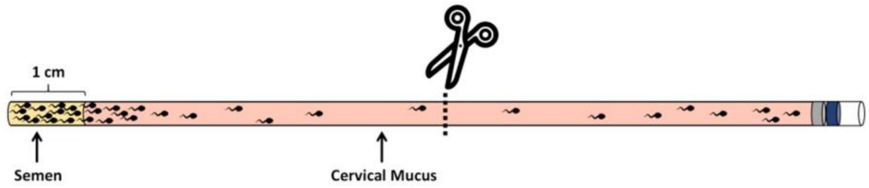
under the microscope using a sperm counting plate, with each sample repeated at least three observation by5 individuals and their evaluation



of the samples.

Figure 2. Schematic diagram of sperm penetration experiment.

Statistical Analysis

The collection volume and type ratio of cervical mucus were statistically analyzed using SPSS (IBM SPSS 25.0, SPSS Inc., Chicago, IL, USA) statistical analysis software with one-way analysis of variance (ANOVA). The scanning electron microscopy results of the cervical mucus were analyzed using Image J software (https://imagej.net/ij/, accessed on 20 July 2024). For the sperm counts after penetration, the ANOVA method in the SPSS sta- tistical analysis software was used to assess the significance of differences between groups.

Results

Observation of Cervical Mucus Crystallization in Dairy Cows during Different Estrous PhasesUsing Optical Microscopy

The quantities collected during each phase are presented in Table 1. During the proestrusphase, cervical mucus was collected from 58 cows; during estrus, from 57 cows; and during the metestrus phase,

times. This study involved simultaneous

from 57 cows. The crystallization of the cervical mucus during the proestrus,estrus, and metestrus phases in dairy cows was observed under an optical microscope. The cervical mucus from all three phases included Types L, S, P, and G (as shown in Figure 3), with varying concentrations of each type during proestrus, estrus, and metestrus (as shown in Table 2). In the proestrus phase, Type L mucus (57.36 ± 5.17%) was significantly more prevalent than Type S (19.42 ± 4.33%), Type P (16.92 ± 4.27%), and Type G (3.55 ± 1.09%)($p < 0.05$). During estrus, Type S mucus (55.82 ± 6.13%) was significantly more abundant than Type L (23.53 ± 4.01%), Type P (18.94 ± 3.25%), and Type G (5.02 ± 1.47%) ($p <$ 0.05). In the metestrus phase, Type P mucus (62.35 ± 5.25%) was significantly more prevalent than Type L (13.66 ± 3.62%), Type S (25.41 ± 4.89%), and Type G (4.85 ± 1.57%) ($p < 0.05$).

Table 1. Cervical mucus collection volumes.

Group	Number of Cows (Head)	Cervical Mucus Collection Volume (mL)
Proestrus	60 (58)	32.55 ± 6.31 [b]
Estrus	60 (57)	59.83 ± 14.45 [a]
Metestrus	60 (57)	27.36 ± 9.92 [b]

The number in parentheses is the number of cows from which samples were collected. Values within the same column with the same superscript letters indicate no significant difference ($p > 0.05$), while values with different superscript letters indicate a significant difference ($p < 0.05$).

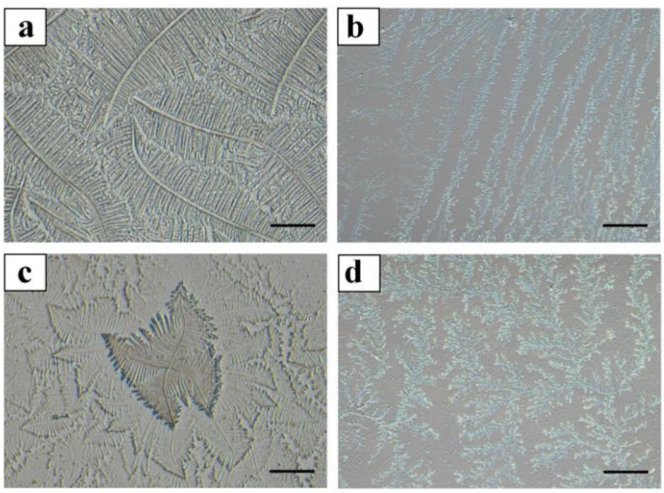


Figure 3. Crystallization patterns of different types of cervical mucus in dairy cows. Note: In Figure 3, panel (**a**) represents Type L cervical

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mucus, characterized by a straight or curved central axis withbranches extending at a 90° angle; panel (**b**) shows Type S cervical mucus, with parallel-aligned crystals featuring short and prominent branches; panel (**c**) depicts Type P cervical mucus, which has two primary axes at a 90° angle to each other; panel (**d**) illustrates Type G cervical mucus, predominantly exhibiting a coral-like structure with no fixed form. Scale bar = 200 μm.

Table 2. Proportion of different types of cervical mucus in dairy cows during proestrus, estrus, andmetestrus.

Group	Number of Cows (Head)	Content of Different Types of Cervical Mucus (%) L	S	P	G
Proestrus	60 (58)	57.36 ± 5.17 ^{a}	19.42 ± 4.33 ^{bc}	16.92 ± 4.27 ^{bc}	3.55 ± 1.09 ^{d}
Estrus	60 (57)	23.53 ± 4.01 ^{bc}	55.82 ± 6.13 ^{a}	18.94 ± 3.25 ^{bc}	5.02 ± 1.47 ^{d}
Metestrus	60 (57)	13.66 ± 3.62 ^{c}	25.41 ± 4.89 ^{b}	62.35 ± 5.25 ^{a}	4.85 ± 1.57 ^{d}

The number in parentheses is the number of cows from which samples were collected. Within the same column,data with the same superscript letters indicate no significant difference ($p > 0.05$), while data with different superscript letters indicate a significant difference ($p < 0.05$).

Scanning Electron Microscopy Observation of Cervical Mucus

The scanning electron microscopy observation of different types of cervical mucus (Types L, S, P, and G) from dairy cows during the proestrus (as shown in Figure 4), estrus, and metestrus phases revealed that the microstructures of the cervical mucus types were consistent across the different estrous phases. The pore sizes of the same type of cervical mucus across different estrous phases showed no significant differences ($p > 0.05$). However,the pore sizes of different types of cervical mucus within the same estrous phase were significantly different ($p < 0.05$), (as shown in Table 3).

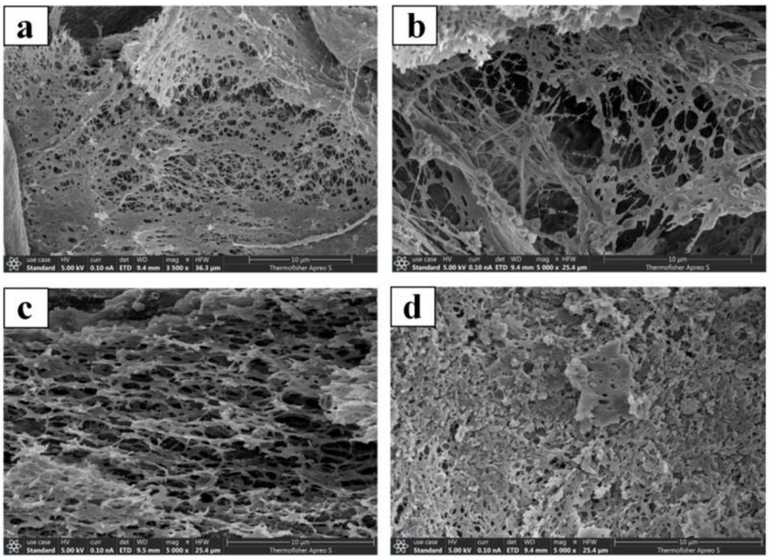

Figure 4. Scanning electron microscopy images of cervical mucus. In Figure 4, panel (**a**) representsType L cervical mucus; panel (**b**) represents Type S cervical mucus; panel (**c**) represents Type P cervicalmucus; panel (**d**) represents Type G cervical mucus.

Table 3. Measurement results of pore sizes of different types of cervical mucus in dairy cows during proestrus, estrus, and metestrus.

roup Number of

Pore Length of Different Types of Cervical Mucus (μm)

Cows (Head) L S P G

1.75 ± 0.86 ^{b} 6.39 ± 2.02 ^{a} 3.57 ± 1.05 ^{ab} 0.42 ± 0.33 ^{c}

Metestrus 60 (57) 1.79 ± 0.72 ^{b} Proestrus 6.44 ± 2.31 ^{a} 60 (58) 3.42 ± 1.26 ^{ab} 1.82 ± 0.75 ^{c} 0.45 ± 0.36 6.50 ± 1.94 ^{a} 3.38 ± 0.95 ^{bc} 0.38 ± 0.29 ^{d} Estrus 60 (57)

The number in parentheses is the number of cows from which samples were collected. Within the same column,data with the same superscript letters indicate no significant difference ($p > 0.05$), while data with different superscript letters indicate a significant difference ($p < 0.05$).

Statistical Results of Sperm Counts Penetrating Cervical Mucus in Dairy Cows duringProestrus, Estrus, and Metestrus

The sperm counts that penetrated the cervical mucus collected through sperm penetra-tion experiments were statistically analyzed (as shown in Table 4). The cervical mucus dur- ing estrus allowed for more sperm

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penetration than the cervical mucus during metestrus, although the difference was not significant, with 68 7.32 million and 58 6.61 million penetrating sperms, respectively ($p > 0.05$). However, both the cervical mucus during estrus and metestrus showed significantly more penetrating sperms than the cervical mucus during proestrus, with 36 ± 5.18 million penetrating sperms ($p < 0.05$).

Table 4. Sperm penetration results in cervical mucus of dairy cows during proestrus, estrus, andmetestrus.

Group	Number of Cows (Head)	Penetrating Sperm Counts (Million/mL)
Proestrus	60 (58)	36 ± 5.18 [c]
Estrus	60 (57)	68 ± 7.32 [a]
Metestrus	60 (57)	58 ± 6.61 [ab]

The number in parentheses is the number of cows from which samples were collected. Within the same column,data with the same superscript letters indicate no significant difference ($p > 0.05$), while data with different superscript letters indicate a significant difference ($p < 0.05$).

Discussion

This experiment used an optical microscope to observe the crystallization of cervi- cal mucus with different shapes, obtaining the same cervical mucus crystals as Vojtěch Pešan [13]. It has been discovered for the first time that the cervical mucus of dairy cowsalso includes four types: L-type, S-type, P-type, and G-type. This experiment used cervicalmucus from the proestrus, estrus, and metestrus phases. Cervical mucus types L, S, P, and G were observed in the cervical mucus of all three estrous phases, indicating that the composition of cervical mucus is roughly the same during different estrous periods. Cervical mucus is mainly composed of these four types [20], with the typical fern-like Type L appearing predominantly near the estrus phase [21]. This is crucial for determining theonset of estrus, and it suggests that the crystalline type of cervical mucus may be related to the timing of ovulation [22]. We also analyzed the proportion of each type of cervical mucus during different estrous phases. Type L cervical mucus was relatively more abundant in the proestrus phase; it was found that L-type cervical mucus is relatively more abundant in the proestrus phase; S-type cervical mucus is relatively more abundant during estrus; and P-type cervical mucus is relatively more abundant in the metestrus phase. The occurrence of this result is due to the control of the branch-like distribution of cervical mucus by estrogen and progesterone, two ovarian hormones. The crystallization of cervical mucus occurs under the influence of estrogen, while progesterone reduces the formation of thebranch-like pattern. Therefore, it is very useful in predicting the onset of estrus, differentstages of estrus, and the timing of ovulation in cattle [23]. This study provides a more comprehensive investigation into bovine cervical mucus.We utilized scanning electron microscopy to observe the microstructure of cervical mucus and conducted pore size analysis, revealing that there is no significant difference in the micro-pore size of the same type of cervical mucus at different stages of estrus. This finding is consistent with the research results of Pilar Vigil and colleagues [15]. We have discovered that there is a significant difference in the micro-pore size of different types of cervical mucus during the same estrous period. Among the four types of cervical mucus, only the S-type cervical mucus has a micro-pore size larger than the width of a sperm head 4~7 μm [15,17], with an average pore size ranging from 4.13~8.75 μm. The pore size of Type P cervical mucus 2.43~4.68 μm was also slightly larger than the width of thesperm head. The microscopic pore size of cervical mucus directly affects the efficiency of sperm penetration. In the sperm penetration experiment, it was found that the highest number of sperm penetrate the cervical mucus during estrus, which is closely related tothe pore size of different types of cervical mucus [24]. Type S cervical mucus is more prevalent in the cervical mucus during estrus, and the average microscopic pore size of Type S cervical mucus is slightly larger than the width of the sperm head, facilitating sperm penetration in the cervical mucus [25,26]. The speed at which sperm swim in cervical mucusis approximately 70~110 μm/s [27]. In the design of this experiment, sperm were allowed to swim in the semen capillary for 30 min, providing them with ample time to swim and facilitating the accurate collection of sperm. During the proestrus, estrus, and

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metestrus phases, the cervical mucus during estrus allowed for the most sperm penetration, whichmay also be related to the cow's ovulation during estrus. The reproductive mechanismof cows leads to an increase in Type S cervical mucus during estrus, and the pore size of Type S cervical mucus is the largest among the four types, thereby increasing the likelihood of sperm penetration. The characteristics of cervical mucus undergo changes during the ovulatory cycle. Near ovulation, due to the gelatinous hydration caused by estrogen, the mucus volume increases, becoming less viscoelastic, thus promoting sperm penetration. In contrast, during the luteal phase, under the influence of progesterone, the mucus becomesa low-water content, highly viscoelastic structure, serving as a barrier to sperm [28].

Conclusions

In this experiment, L-type, S-type, P-type, and G-type cervical mucus were observed in cervical mucus during the proestrus, estrus, and metestrus phases, with different pro- portions of these four types during different estrous phases. The microscopic pore sizes of L-type, S-type, P-type, and G-type cervical mucus also varied, with S-type cervical mucus having the largest pore size, with an average pore size ranging from 4.13 to 8.75 μm. The sperm penetration effects of cervical mucus during different estrous phases also differed, being closely related to the content of S-type cervical mucus.

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Author Contributions: F.H., L.-L.Z., Q.-H.G. conceived, drafted, and approved this research article.Conceptualization: Q.-H.G.; data curation: F.H.; formal analysis: P.N.; funding acquisition: Q.-H.G., F.H., L.-L.Z.; methodology: Q.-H.G., F.H., D.F., J.-J.S.; resources: F.H., P.N., J.W., L.-L.Z. visualization: F.H., X.-P.L.; roles/writing—original draft: F.H., J.-R.W.; writing—review & editing: Q.-H.G., X.-Y.W.,L.-L.Z. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal experiments were conducted in accordance with the "Regulations and Guidelines for the Management of Experimental Animals" established by theMinistry of Science and Technology (Beijing, China, 2020 revision). This study was approved by the Institutional Animal Care and Use Committee of Tarim University, Xinjiang, China (protocol code DWBH20220101; approval date: 1 January 2022).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare that there are no competing interests associated with the manuscript.

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