Short Paper

Cervical mucus characteristics and hormonal status at insemination of Holstein cows

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Summary

The present study was carried out to characterize the cervical mucus (CM) collected when inseminating Holstein cows and to relate the secretion pattern with pregnancy. The mucus was collected from mid-cervix of 64 cows with spontaneus estrus (SE) and induced estrus (IE). The quantity, pattern and consistency of the mucus, pH, arborization patterns and the sperm motility were observed. The levels of progesterone and estradiol 17β in serum were also determined. Pregnancy detection was performed by means of transrectal ultrasonography 60 days after insemination. Mucous secretion of cows with SE was significantly different from those with IE, showing a lower degree of crystallization (SE 2.00; IE 2.75) and a lower level of steroid hormones (P4: SE 0.17 ng/ml and IE 0.33 ng/ml (t 1.99547 α 0.05); E2: SE 30.95 pg/ml and IE 47.76 pg/ml (t 1.99495 α 0.05). Progesterone level was significantly lower and estrogen significantly higher in pregnant cows (P) in relation to that observed in non pregnant females (N) (P4: P 0.20 ng/ml and N 0.44 ng/ml (t 1.99602 α 0.05); E2: P 54.77 pg/ml and N 40.75 pg/ml (t 1.99505 α 0.05). In conclusion pregnancy was associated with acopious, clear and watery discharge (similar to egg white), with an arborization degree of 2.25, presence of atypical fern leaves together with rosette formations and needles or thorns on rails due to low levels of progesterone accompanied by high concentrations of estrogens.

Key words: Cristalization, Estrogen, Insemination, Pregnancy, Progesterone

Introduction

The individual production of dairy cows has increased significantly over the last 40 years and in most countries - like Argentina - individual production has almost doubled its level, due to a combination of genetic, feeding and handling improvements. However, this high productivity occurred along with a global decrease in reproductive efficiency of dairy cattle (capacity of the female to produce a living calf), particularly in the world dominant breed: Holstein (Marini and Oyarzabal, 2002a, b; Chagas *et al*., 2007). The possibility of a lower reproductive efficiency had already been announced by Gaines (1927), when stating that lactation affected the reproductive cycle of some animals and that ensuring a really high level of production would interfere with conception.

We consider that the cervical mucus (CM) characteristics play a crucial role in conception. The properties or characteristics of cervix secretion, as well as its quantity, vary according to the hormone predominance which corresponds to the stage of the estrus cycle (Moghiss and Blandau, 1972). Its evaluation may be indirectly used as an indicator of the stage of the cycle or the female hormonal condition in most domestic animals and humans (Benbia *et al*., 2011). It has been proved through many studies that there is a strong interaction between the CM and the sperm and that its properties may empower or impede sperm motility, even when the sperm can avoid the cervix as is the case with artificial insemination (AI), influencing fertility (Gaddum-Rosse, 1981; Yousef, 1981). In this sense the aim of this study was to characterize cervical mucus macroscopically and microscopically at insemination, to evaluate sperm penetration and to detect serum estradiol-17β and progesterone levels in cows with natural as well as induced estrus, and in pregnant and nonpregnant heifers.

Materials and Methods

Animals

Holstein cows (n=64) in good body condition and with anatomically normal genitals were studied according to the type of estrus: SE (spontaneous estrus) and IE (induced estrus). SE cows (n=23) were inseminated when spontaneous estrus was detected and in IE cows (n=41) ovulation was synchronized and hormonally induced, and they were subsequently inseminated. Spontaneous estrus was detected by highly trained people through direct observation of animal behavior twice a day (morning and evening) and through

the observation of specific characteristics of the first signs of estrus.

The estrus synchronization protocol used in IE was as follows: 2 mg estradiol benzoate (EB) were IM injected on day 0 – the day when the intravaginal progesterone releasing device was inserted (1 g P4, DIB, Syntex, Argentina). On day 7 or 8 the device was removed and progesterone and prostaglandine PGF (150 g D (+) cloprostenol, Ciclase, Syntex) and 1 mg estradiol cypionate (Cypiosin, Syntex SA, Argentina) were administered in order to synchronise ovulation.

Artificial insemination (AI) was carried out 54 or 56 h after device removal. All cows were inseminated with frozen semen which had been proved to be fertile. Pregnancy was detected through transrectal ultra-sonography 60 days after insemination.

Cervical mucus (CM)

Samples of CM were collected immediately before AI by gentle aspiration from the cervix and stored at -20°C for later use.

Macroscopic description

The parameters used were: quantity (copious, moderate, absent), appearance (clear, cloudy and dirty) and consistency (thick, medium, watery) according to Deo and Roy's classification (1971). The pH was measured using reactive paper (range 0-10) *in situ* at insemination.

Microscopic description

The arborization pattern was ranked in a 0 to 4 scale (Tsigilianni *et al*., 2011). Measures are shown in Fig. 1 and expressed in micrometers. Software Image J 1.45 was used.

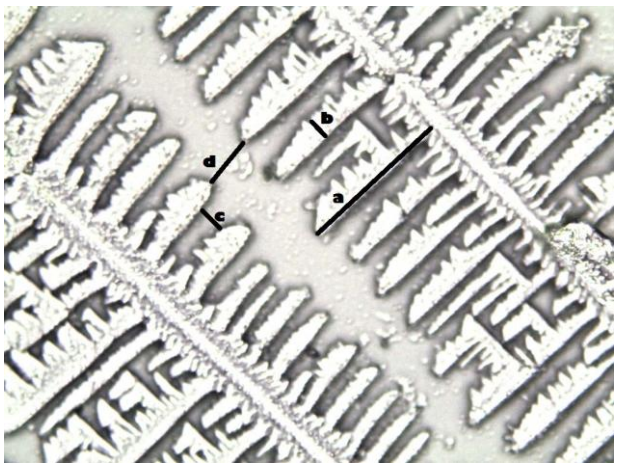


Fig. 1: Dimensions assessed on arborizations in cervical mucus. a: long secondary sheet (I), b: sheet width, c: space between sheets, and d: distance among filaments or primary leaves, (×400)

Sperm penetration

An *in vitro* test known as the simplified slide test was carried out, and the classification was: normal, poor, subnormal and negative (OMS, 2001).

Blood samples

Blood samples were collected at AI (10 ml/animal). Serum was separated and stored at -20°C. The estradiol 17β (E2, pg/ml) and progesterone (P4, ng/ml) concentrations were determined through electro-chemiluminescence (EQLIA) (ALKEMY Lab Center, Argentina).

Statistical analysis

Student's t-test was used to check whether there were differences in both groups: spontaneous and induced estrus; as well as pregnant and empty cows. Contingency test was used to compare macroscopic parameters and sperm penetration. The correlation coefficients were estimated:

a) Between the concentration of estrogen (E2) and progesterone (P4)

b) Between estrogen concentration and the degree of crystallization

c) Between progesterone concentration and the degree of crystallization

d) Degree of crystallization and sperm penetration in both groups (SE and IE) and for pregnant (P) and open or non-pregnant (NP) cows.

Results

The macroscopic characteristics of the CM and the frequency of its appearance at insemination are shown in Table 1.

Table 1: Frequency of occurrence of bovine cervical mucus at insemination (%)

Characteristics of CM		SE %	IE %	P %	NP %
Quantity	Abundant	56	36	44	17
	Moderate	31	44	35	33
	Low/Absent	13	20	21	50
	100%				
Appearance	Transparent	65	56	70	45
	Opaque	35	43	30	54
	Unclean	0	1	0	1
	100%				
Consistency	Dense	13	32	9	50
	Medium	31	39	30	17
	Liquid	56	29	61	33
	100%				

CM: Cervical mucus, SE: Spontaneous estrus, IE: Induced estrus, P: Pregnant, and NP: Non-pregnant

Arborization of CM varied widely within females, it did not differ between groups (spontaneous estrus vs. induced estrus). The atypical fern pattern is observed in Figs. 2A-B, in Fig. 3 shows the typical leaves which were placed in a sort of channel (Fig. 4). Both formations (typical and atypical fern) correspond to mucus type L (*loaf*). Also, rosette or star formations with 4 or 6 axes coming out of the centre in a 45° or 60° angle were

observed, their forms correspond to mucus type P (peak) (Figs. 5A-C). Only in a few cases were short branched crystals observed as needles set along an axis or rail and these were arranged in parallel, these are mucus type S (*string*) (Fig. 6).

A

B

Fig. 2: Crystallization of cervical mucus in atypical fern leaves. Different forms of atypical leaves related to liquid mucus, (×100). A: Spontaneous estrus, and B: Induced estrus

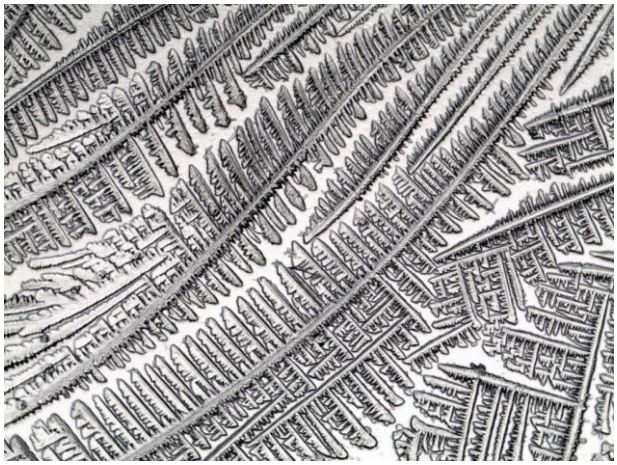


Fig. 3: Crystals of bovine cervical mucus arranged in typical fern leaves (induced estrus) (×100)

The results obtained from measuring typical and atypical ferns observed in the watery and thick CM, are shown in Table 2. Statistically significant differences ($P<0.001$) appeared when observing the size of crystals in thick mucus in contrast to the watery one. In order to find differences in the CM of cows with natural and induced estrus, the pH value, crystalization degree and penetration of spermatozoa into the CM as well as the level of serum progesterone and estrogen were compared (Table 3).

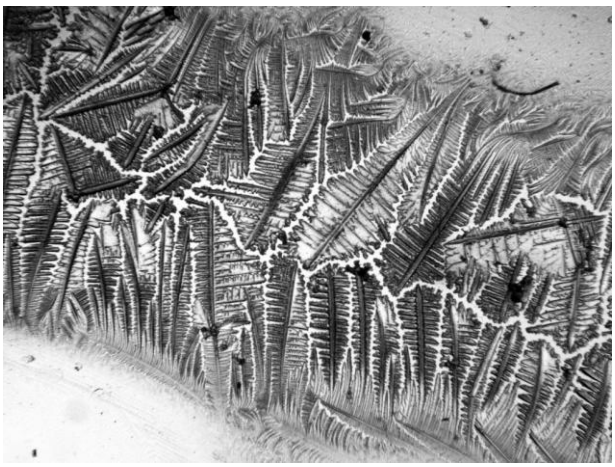
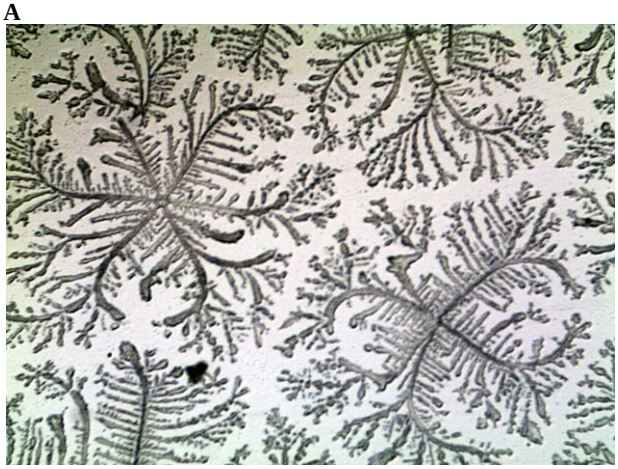
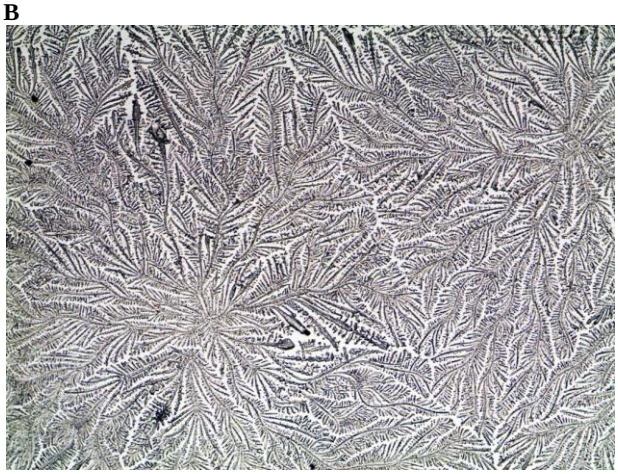


Fig. 4: Arrangement of typical shaped crystals observed fern in smears of bovine cervical mucus (spontaneous estrus) (×40)

A



B



C

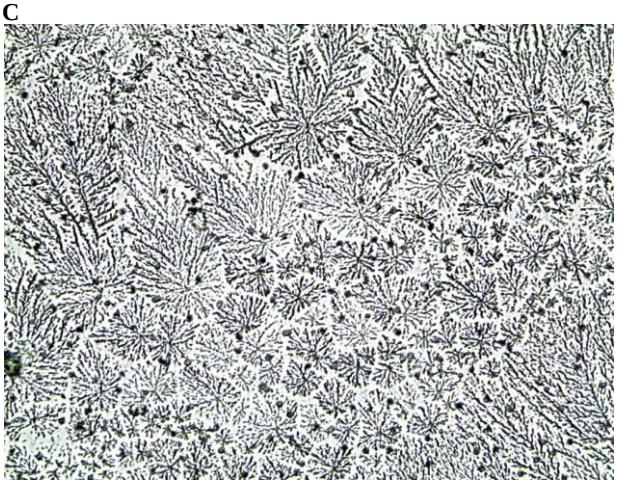


Fig. 5: Crystal formations as a star. Bovine cervical mucus. A and C: Spontaneous estrus, and B: Induced estrus, (×100)

Fig. 6: Pattern shaped crystals short parallel needles (induced estrus), (×100)

In the case of animals with IE (pregnant and non-pregnant) Table 1 shows the macroscopic characteristics observed on the MC and, in Table 4, the microscopic features and estrogen and progesterone levels are shown.

A statistically significant positive correlation between P4 and E2 levels was observed in both pregnant (P) and non-pregnant cows (NP); and an inverse correlation between the degree of crystallization and penetration of spermatozoa was also observed in both groups (P4-E2: 0.83 (P), 0.78 (NP); CR-PE: -0.61 (P), -0.69 (NP)). The same situation was found when analyzing the SE groups and IE (P4-E2: 0.59 (SE), 0.78 (IE); CR-PE: -0.86 (SE), -0.65 (IE)).

Table 2: Dimensions corresponding to the fern leaves (μm)

Parameter	SE (X+SE)	IE (X+SE)
CM dense		
Leaf length	495.11+56.40[a]	489.66+39.02[a]
Leaf width	97.36+13.99[a]	96.52+9.36[a]
Spacing sheet	12.01+1.65[a]	15.85+1.57[a]
Distance between filament	22.20+1.10[a]	29.14+1.05[a]
CM liquid		
Leaf length	336.59+39.02[b]	335.19+55.03[b]
Leaf width	39.50+4.42[b]	37.02+55.03[b]
Spacing sheet	23.99+3.14[b]	29.79+3.42[b]
Distance between filament	125.74+1.34[b]	156.03+1.17[b]

Different letters indicate significant differences column by parameter (P<0.001). CM: Cervical mucus, SE: Spontaneous estrus, and IE: Induced estrus

Discussion

Copious, transparent and watery mucus was frequently present in cows with natural estrus and its degree of arborization turned to be significantly lower than the one observed in the secretion of cows with induced estrus. Pregnancy was associated to cows whose mucus appeared transparent, filant (between watery and biscous) and in considerable quantity (between moderate and copious), typical characteristics of egg white. We observed that pregnancy was associated with mucus that is visually filant and clear, since such mucus was observed in 65% of the pregnant cows. Cows with thick mucus resulted in open or empty (60%). This CM appearance indicates good sexual health of the animal and is also associated with non-newtonian properties, behaviour that was associated with a higher conception rate when this mucus secretion was present at insemination (Murugavel and López-Gatius, 2009).

Transparent crystallized fluid mucus with a low degree of arborization (between 1 and 2) boosted sperm motility through the mucus. By contrast, thicker and opaque crystallized secretion - higher grade (3 to 4) -

Table 3: Mean and standard error for pH, degree of crystallization of the cervical mucus, sperm penetration and serum estrogen (E2) and progesterone (P4). Spontaneous estrus (SE) and induced (IE)

Estrus	pH[1]	Crystallization (0-4)[1]	P4 (ng/ml)[1]	E2 (pg/ml)[1]	Sperm penetration (%)[2]
SE	7.0 + 0.0[a]	2.00 + 0.18[a]	0.17 + 0.03[a]	30.95 + 2.52[a]	Normal 88.45[a]
IE	7.4 + 0.1[a]	2.75 + 0.19[b]	0.33 + 0.02[b]	47.76 + 3.75[b]	Normal 78.58[a]

[1] Student's t-test, and [2] Test of contingency. Different letters indicate significant differences column by parameter (P<0.05)

Table 4: Mean and standard error for pH and degree of arborization of cervical mucus penetration sperm in the mucus. Induced estrus: pregnant and nonpregnant cows

Reproductive status	pH[1]	Crystallization (0-4)[1]	P4 (ng /ml)[1]	E2 (pg /ml)[1]	Sperm penetration (%)[2]
Pregnant (n=18)	7.6 + 0.1[a]	2.25 + 0.12[a]	0.20 + 0.03[a]	54.77 + 6.26[a]	Normal 87.53[a]
Nonpregnant (n=23)	7.2 + 0.1[a]	3.25 + 0.15[b]	0.44 + 0.04[b]	40.75 + 4.49[b]	Normal 69.64[b]

[1] Student's t-test, and [2] Test of contingency. Different letters indicate significant differences column by parameter (P<0.05)

complicated the progression of spermatozoa. It is well known that the characteristics of cervical mucus are modified according to the function of ovarian hormones secreted during estrus, and this mucus can therefore act either as a mechanical barrier to sperm motility, or as a facilitator of its way to the ovule and allow its fertilization (Tsiligianni *et al*., 2011; Layek *et al*., 2013).

In our study, the serum concentration of progesterone was always low (minimum average value 0.17 ng/ml; maximum average value 0.44 ng/ml), i.e. at levels compatible to a follicular phase of the cycle; and the level of estrogen was high (minimum average value 30.95 pg/ml; maximum average value 54.77 pg/ml), a concentration which might be considered as part of the pre-ovulatory wave (Tsiligianni *et al*., 2011). No significant differences were found among animals in relation to their estrus, although there were differences between pregnant and empty cows. Pregnancy was related to a lower P4 concentration, higher level of E2 and therefore a lower degree of crystallization and better sperm penetration. These results could be explained since at insemination, the cow was in estrus, a time when there is lack or recession of the corpus luteum; facts which are in agreement with the low levels of progesterone detected, and which favour the final follicular development (Predojevic *et al*., 2007). As regards estrogen, its high level is essential for reproductive success, since it is supposed to contribute to the production of the LH pulse within the next few hours which would allow the subsequent ovulation (Tsiligianni *et al*., 2011).

In all cases the pH value of the CM ranged from 7.00 to 7.60 and no significant differences were found when comparing cows with natural and induced estrus, in agreement with the reports made by Tsiligianni *et al*. (2001), nor between pregnant and empty cows.

In conclusion, the visual appearance of the cervical mucus can be used as another tool to be considered in order to improve estrus detection and determine the right moment to inseminate, always looking forward to achieving a higher percentage of pregnancy. Fertility was associated with an arborization degree between 1 and 2, atypical fern like formations together with rosettes and needle-or-thorn like forms on rails (mucus type L, S and P). We consider that further studies would contribute to explaining the lack of homogeneity of mucus discharge in cows when estrus is synchronized, independently of the protocol used. These will further contribute to update or modify the insemination practice at an exact periovulatory period achieving greater rate of pregnancy.

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Conflict of interest

The authors state that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

References

Benbia, S; Kalla, A; Yahia, M; Belhadi, K and Zidani, A (2011). Enzymes activity in bovine cervical mucus related to the time of ovulation and insemination. International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering. 5: 664-666.

Chagas, L; Bass, J; Blache, D; Burke, C; Kay, J; Lindsay, D; Lucy, M; Martin, G; Meier, S; Deo, S and Roy, D (1971). Investigations on repeat breeding cows and buffaloes studies on physical properties of cervical mucus. Indian Vet. J., 48: 479-484.

Gaddum-Rosse, P (1981). Some observations on sperm transport through the uterotubal junction of the rat. Am. J. Anat., 160: 333-341.

Gaines, WL (1927). Milk yield in relation to recurrence of conception. J. Dairy Sci., 10: 117-125.

Layek, S; Mohanty, T; Kumaresan, A; Behera, K and Chand, S (2013). Cervical mucus characteristics and periestrual hormone concentration in relation to ovulation time in Zebu (Sahiwal) cattle. Livest. Sci., 152: 273-281.

Marini, P and Oyarzabal, M (2002a). Patrones de producción en vacas lecheras. I Componentes de la producción y sus compartamientos según el nivel de producción. Rev. Arg. Prod. Anim., 22: 29-46.

Marini, P and Oyarzabal, M (2002b). Patrones de producción en vacas lecheras. II Descripción de la vaca media y estimación de los ingresos según categorías de producción. Rev. Arg. Prod. Anim., 22: 47-60.

Murugavel, K and López-Gatius, F (2009). Newtonian behavior of the vaginal fluid as a risk indicator of reduced fertility in cows. Indian Vet. J., 86: 1288-1289.

Organización Mundial de la Salud (OMS) (2001). In: Handbook of WHO Laboratory for examination of human semen and interaction between semen and cervical mucus. 4ª ed., Ed. Médica Panamericana SA., Madrid, España. PP: 70-72.

Predojevic, RM; Petrujkic, T; Petrujkic, TB and Predojevic, MN (2007). Influence of the ovarian hormones on the cervical mucus (biophysical properties) and sperm transport in relation to cow's conception rates. Lucr. Şt. Med. Vet. Timişoara. XL: 91-94.

Tsiligianni, Th; Amiridis, G; Dovolou, E; Menegatos, I; Chadio, S; Rizos, D and Gutierrez-Adan, A (2011). Association between physical properties of cervical mucus and ovulation rate in superovulated cows. Can. J. Vet. Res., 75: 248-253.

Tsiligianni, Th; Karagiannidis, A; Brikas, P and Saratsis, Ph (2001). Physical properties of bovine cervical mucus during normal and induced (progesterone and/or PGF2α) estrus. Theriogenology. 55: 629-640.

Yousef, A (1981). Note on the biochemical aspects of bovine cervical mucus. Indian J. Anim. Sci., 51: 1082-1085.