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HIGHLY SYMMETRICAL CRYSTALLIZATION IN SIX RECTILINEAR AND WELL-DEFINED AXES FOUND IN BOVINE CERVICAL MUCUS OBTAINED AT OESTRUS: A FINDING

*M. E. Cortés[1,2,3,4 *], F. González[3], R. Hauyón[4], P. Vigil[4,5,6]*

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ABSTRACT

Bovine cervical mucus (BCM) is important for selection and transport of spermatozoa. When air-dried, BCM obtained at oestrus exhibits arborescent crystallizations, among other arrangements. Considering the relevant endocrine and reproductive information indirectly obtained from BCM crystallization, a morphological investigation was carried out to study its crystalline patterns. BCM samples were collected from healthy Holstein Friesian heifers at oestrus, their crystalline patterns photographed and its morphology analyzed. The majority of the crystallizations obtained showed the typical tree-like patterns reported for BCM. However, a highly symmetrical arrangement was found, characterized by a star-like morphology with six straight, highly defined axes that protrude from the same central point, forming 60º angles. In terms of current knowledge, this short report is the first to show this crystallization geometry in BCM, which, additionally, is remarkably similar to $P6_B$ mucus reported for periovulatory human cervical mucus. Even though the role of mucus presenting this type of crystallization is as yet unknown for bovines, its possible functions are also briefly discussed here.

Key words: Bovine cervical mucus, crystallization, oestrus, $P6_B$ mucus.

CRISTALIZACIÓN ALTAMENTE SIMÉTRICA CON SEIS EJES RECTILÍNEOS Y BIEN DEFINIDOS HALLADA EN MOCO CERVICAL BOVINO OBTENIDO EN ESTRO

RESUMEN

El moco cervical bovino es importante para la selección y el transporte espermático. El moco, obtenido durante el estro y secado al aire, exhibe cristalizaciones con formas

1 Departamento de Ciencias Químicas y Biológicas, Universidad Bernardo O'Higgins, General Gana 1702, Santiago (Chile).

2 Escuela de Kinesiología, Facultad de Salud, Deporte y Recreación, Universidad Bernardo O'Higgins, General Gana 1702, Santiago (Chile).

3 Departamento de Ciencias Animales, Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile, Av. Vicuña Mackenna 4860, Macul, Santiago (Chile).

4 Reproductive Health Research Institute, Lira 140, of. 201, Santiago (Chile).

5 Pontificia Universidad Católica de Chile, Alameda B. O'Higgins 340, Santiago (Chile).

6 Fundación Médica San Cristóbal, Av. Luis Pasteur 5292, Vitacura, Santiago (Chile).

* Autor para correspondencia: manuel.cortes@ubo.cl / manuelcortes@uc.cl

principalmente arborescentes. Considerando la importante información endocrina y reproductiva que es posible obtener a partir de la cristalización del moco cervical, se efectuó una investigación morfológica con el propósito de estudiar sus patrones cristalinos. Las muestras de moco se obtuvieron de novillas Holstein Friesian en estro; posteriormente, los patrones de cristalización del moco fueron fotografiados para finalmente analizar su morfología. Las cristalizaciones obtenidas correspondieron a típicos patrones arboriformes previamente reportados. Sin embargo, lo que llamó la atención fue el hallazgo de un arreglo altamente simétrico en una novilla, caracterizado por una morfología similar a estrella con seis ejes rectos, bien definidos, que surgen desde el mismo punto central y forman ángulos de 60º. Según nuestro conocimiento, esta comunicación breve reporta por primera vez la presencia de dicha geometría de cristalización en vaquillas, la cual es muy semejante al patrón cristalino subtipo $P6_B$ reportado para el moco cervical periovulatorio humano. Si bien el rol ejercido por este tipo de cristalización de moco aún se desconoce en bovinos, se discuten aquí sus posibles funciones.

Palabras clave: moco cervical bovino, cristalización, estro, moco $P6_B$.

INTRODUCTION

Since ancient times, nomad tribes have recognized the periodic discharge from the reproductive tract of their herds, understanding this as a visible sign of the beginning of oestrus, and thus, of sexual receptivity (Pommerenke 1962). This substance is known as mucus or cervical secretion and is found in ruminant species such as bovine and ovine (Bone 1954; Chargoy 2001; Rutllant *et al.* 2005; Richardson *et al.* 2011), as well as in other species of mammals, such as humans (Pommerenke 1962; Odeblad 1994; Menárguez 1998). Bovine cervical mucus (BCM) is produced by the mucus-secreting epithelial cells located in the endocervix. From a biochemical point of view, the BCM is a hydrogel constituted by an aqueous phase in which several low-molecular-mass compounds (e.g., ions and metabolites) are dissolved, and a gel phase formed mainly by mucins, high-molecular-mass and highly glycosylated glycoproteins which give mucus its characteristic rheological properties (Schumacher 1973; Rutllant *et al.* 2005; Vigil *et al.* 2009). Among the functions of cervical mucus, the most relevant might be its role in sperm selection and transport, being the first medium spermatozoa goes through in the female reproductive tract when ascending towards the site of fertilization (Menárguez 1998; Rutllant *et al.* 2005; Vigil *et al.* 2009). The biophysical and biochemical properties of BCM change during the oestrous cycle (Rutllant *et al.* 2005). Mainly the changing levels of oestrogens and progesterone cause these fluctuations during the cycle (Rutllant *et al.* 2005). BCM is secreted throughout the cycle, but its volume increases at oestrus due to the oestrogenic influence, which also generates the crystallization of the mucus in tree-like patterns resembling fern fronds, palm and pine branches (Bone 1954; Noonan *et al.* 1975; Chargoy 2001; Cortés 2012; Cortés *et al.* 2012; Cortés *et al.* 2014), among other geometric arrangements. Such tree-like crystallization patterns of BCM, related to the ovarian activity of the female (Alliston *et al.* 1958), are present in a higher proportion at oestrus than in any other phase of the cycle (Noonan *et al.* 1975).

Considering the important endocrine and reproductive information obtained from the analysis of cervical mucus, in the past few years our research group has been performing ultrastructural and morphological studies of the characteristics of crystallization patterns of human (Ceric *et al*. 2005; Vigil *et al*. 2009) and bovine cervical secretion (Cortés 2012, Cortés *et al*. 2012). In view of the above, this brief report aims to communicate the finding of a highly symmetrical crystallization pattern forming six rectilinear and well-defined axes in BCM obtained at oestrus; a pattern that shows a striking resemblance to a subtype of mucus crystallization reported in humans.

MATERIALS AND METHODS

Animals studied

The study initially involved 10 reproductively healthy Holstein Friesian heifers (15 months old) from the herd at the Experimental Station of the Faculty of Agronomy and Forestry Engineering, Pontificia Universidad Católica de Chile at Pirque, Región Metropolitana, Santiago, Chile. These heifers were frequently assessed as regards the characteristics of their cervical mucus, according to the requirements of ongoing research at that university experimental station.

Collection of mucus samples

BCM samples were obtained the same day from heifers at oestrus stage, in a previously synchronized heat. Oestrus was confirmed by palpation of the genital organs per rectum as reported by other researchers (López-Gatius y Camón-Urgel 1991). BCM samples were collected by using a 50-mL sterile plastic tube and stored at 4ºC for later analysis (the interval between sampling and analysis was less than 24 h). BCM samples were subsequently analyzed in the laboratory, where those showing opacity, blood content or contamination were discarded. This study considered all aspects concerning animal welfare and the approval of the respective bioethical committee. In addition, the process of mucus sample collection was under permanent supervision of an experienced veterinary doctor.

Analysis of mucus crystallization

The patterns of BCM crystallization were analyzed by smearing a drop of mucus sample on a slide in all directions using a needle according to the 'spread-out' technique (Odeblad 1995; Menárguez 1998); no staining was used in the procedure. Then, the mucus on the slides was air-dried at room temperature (20–25ºC) for at least 15 min before the study, as previously reported in other work (Cortés *et al*. 2012). Finally, the air-dried mucus preparations were observed according to standard procedure at 100×, 200×, 400× and 1000× using an Olympus CK X41 light microscopy, and then photographed.

RESULTS

Most BCM crystallizations obtained presented the typical arrangements including a combination of shapes resembling fern fronds, palm or pine branches (not shown here), which have been widely reported both by other researchers (Bone 1954; Noonan *et al.* 1975; Chargoy 2001; Saba *et al.* 2010) and by our previously published works (Cortés 2012; Cortés *et al.* 2012). However, what really captured our attention was the finding of a highly symmetrical crystallization in a sample obtained from one of the heifers; the pattern was characterized by a star-like morphology

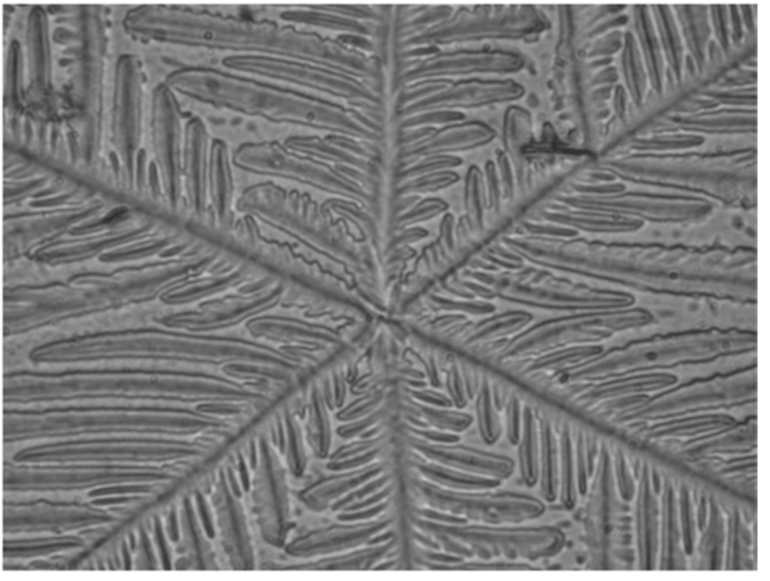

FIGURE 1. Highly symmetrical crystallization for BCM obtained from Holstein heifer at oestrus (light microscopy, 400×). This crystallization is large and characterized by a star-like morphology with six rectilinear and well-defined axes (hexagonal symmetry), which originate from the same central point forming 60° angles between them. From each axis, branches of variable length protrude forming angles of approximately 60° in relation to the axis. The geometric arrangement of this crystalline pattern is very similar to P6B crystallization subtype reported for human cervical mucus.

with six straight and well-defined axes, originating from the same central point and forming 60º angles. From each axis, variable length branches protrude off in approximately 60º angles (Figure 1). This crystallization pattern was found only in one of the 10 heifers whose mucus was originally obtained.

DISCUSSION

The reported crystalline pattern could be considered to be the result of an artifact originating in mucus storage or air-drying procedures; however, this particular crystallization does not constitute an artifact since a similar crystalline structure has been described in a recent study also carried out in heifer cervical mucus (Cortés 2012). This finding captured our attention since, to the best of our knowledge, this is the first time that a geometrical star-like pattern has been reported for BCM crystallization showing such straight axes and such strikingly symmetrical structure, in an arrangement which is also considerable in size. This fact shows that BCM at oestrus, not only crystallizes in the typical geometrical pattern resembling fern fronds, palm or pine branches, as reported by other works (Bone 1954; Noonan *et al*. 1975; Chargoy 2001; Cortés *et al*. 2012), but that other types of geometry for mucus crystallization would also exist. Thus, BCM at oestrus is probably a heterogeneous morphological entity formed by several subtypes of secretion, whose contribution varies, being synthesized in different areas of the cervical epithelium, as suggested for women (Menárguez *et al*. 2003), a fact which would explain the different geometrical patterns found in BCM crystallization. Nevertheless, further studies are necessary in order to investigate the existence of such subtypes and determine their eventual functions in bovine reproductive physiology.

Reviewing literature on cervical mucus we found that, interestingly, the crystallization pattern shown here (see Figure 1) has an astonishing resemblance with $P6_B$ mucus crystallization[7] reported for human cervical mucus, which also possesses a star-like morphology, with six well-defined axes originating from a central point (Odeblad 1994; Menárguez 1998; Vigil *et al*. 1999; Vigil *et al*. 2009; Vigil *et al*. 2012). Geometrically, one would assume Figure 1 to be located in the x-y plane, and by simple inspection, argue that if it were rotated clock- or counter-clockwise to $\pi/3$ radians (i.e., 60º), an image with visually identical properties (i.e., with fractal attributes) would be obtained. That is, after deciding the direction of the rotation, the figure can be rotated 1, 2, 3, 4, 5, and even 6 times, and for each rotation, a highly similar image to the original would be obtained. From the point of view of Theory of Rotational Groups, this would correspond to hexagonal rotation symmetry of order 6 (Wood 1964; Nussbaum 1974).

As regards BCM, to date, there is no satisfactory classification to categorize the different crystallization patterns that can be observed during the oestrous cycle, and specifically in the oestrus stage. This is not the case for women, for whom a model to classify cervical mucus crystallizations has been proposed, known as Odeblad's model (Odeblad 1994; Menárguez *et al*. 2003; Vigil *et al*. 2012). It is known that $P6_B$ mucus crystallization tends to form somewhat larger units than other subtypes of P mucus (Menárguez 1998) and this mucus subtype has been associated to the peak of fertility in humans (Vigil *et al*. 2012), and would be involved in the selection of the spermatozoa that ascend along the cervix (Odeblad *et al*. 2006). Probably, mucus whose crystalline pattern is shown here has similar functions to human $P6_B$ mucus, but its specific reproductive significance in bovines is yet unknown.

[7] Subtype $P6_B$ mucus receives its name since it is associated to the fertility **p**eak, **6** axes characterize its symmetry, and its structure is **b**ig in comparison to other subtypes of mucus crystallizations.

CONCLUDING REMARKS

To the best of our knowledge, this brief communication is the first to report the finding of a highly symmetrical crystalline pattern among the crystallizations of BCM at oestrus, which is characterized by a considerable size and a star-like morphology with six well-defined straight axes (hexagonal symmetry), emerging from the same central point and forming 60º angles. Remarkably, this crystalline pattern is very similar to what has been reported for human $P6_B$ mucus crystallization (see Figure 1). Further research is necessary to determine if the subtype of BCM whose crystalline pattern is reported here exerts any relevant role in the reproductive process of bovines, as has already been proposed in humans.

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Conflict of interest statement

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the manuscript.

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