

**Highly symmetrical crystallization in six rectilinear and well-defined axes**

**found in bovine cervical mucus obtained at oestrus: A finding**

**Hallazgo de una cristalización altamente simétrica con seis ejes rectilíneos y bien definidos**

**encontrada en moco cervical bovino obtenido en estro**

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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 arboriform 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 60° angles. In terms of our knowledge,

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**MANUSCRITO ACEPTADO**

this short report is the first to show this crystallization geometry in BCM, which, additionally, is remarkably similar to P<sub>6B</sub> 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; P<sub>6B</sub> mucus.

**RESUMEN**

El moco cervical bovino es importante para la selección y el transporte espermático. El moco obtenido en estro y dejado secar al aire exhibe cristalizaciones con estructuras principalmente arborescentes. Considerando la importante información endocrina y reproductiva obtenida 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 vaquillas (novillas) Holstein Friesian en estro; posteriormente los patrones de cristalización del moco fueron fotografiados para finalmente analizar su morfología. Las cristalizaciones obtenidas correspondieron a los típicos patrones arboriformes previamente reportados. Sin embargo, lo que llamó realmente la atención fue el hallazgo en una vaquilla de un arreglo altamente simétrico, caracterizado por una morfología similar a estrella con seis ejes rectos, bien definidos, que surgen desde el mismo punto central y forman ángulos de 60°. Según nuestro conocimiento, esta comunicación breve reporta por primera vez la presencia en vaquillas de dicha geometría de cristalización, la cual es muy semejante al patrón cristalino subtipo P<sub>6B</sub> 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:** cristalización, estro, moco cervical bovino, moco P<sub>6B</sub>.

## **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 bovines and ovines (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 go 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 vary during the oestrous cycle (Rutllant et al. 2005). These fluctuations are caused mainly by the changing levels of oestrogens and progesterone 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 arboriform 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 arboriform-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.

## **MATERIAL AND METHOD**

### **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 & Camón-Urgel 1991). BCM samples were collected by using a 50-mL sterile plastic tube and stored at 4°C 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 (20–25°C) 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 with six straight well-defined axes, originating from the same central point and forming 60°

angles. From each axis, variable length branchings protrude in approximately 60° angles (Fig. 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 (Fig. 1) has an astonishing resemblance with P<sub>6B</sub> mucus crystallization<sup>7</sup> 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 Fig.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., 60°), 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 an 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 P<sub>6B</sub> 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 P<sub>6B</sub> mucus, but its specific reproductive significance in bovines is yet unknown.

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<sup>7</sup> Subtype P<sub>6B</sub> mucus receives its name since it is associated to the fertility peak, its symmetry is characterized by 6 axes and its structure is big 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 60° angles. Remarkably, this crystalline pattern is very similar to what has been reported for human P6<sub>B</sub> mucus crystallization (Fig. 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.

### **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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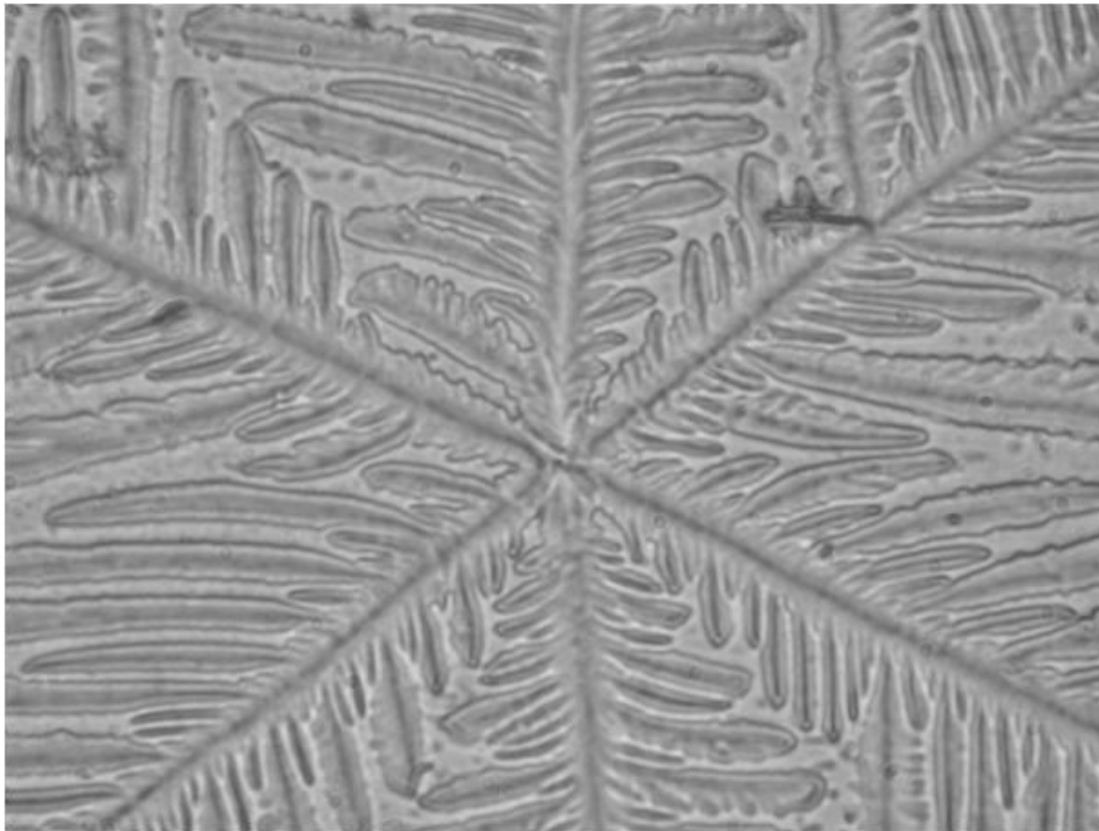


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**Figure 1. Highly symmetrical crystallization for BCM obtained at oestrus.** This crystallization is large and characterized by a star-like morphology with six rectilinear and well-defined axes (hexagonal symmetry) which originate from the same central point forming  $60^\circ$  angles between them. From each axis, branchings of variable length protrude forming angles of approximately  $60^\circ$  in relation to the axis. The geometric arrangement of this crystalline pattern is very similar to  $P6_B$  crystallization subtype reported for human cervical mucus. Light microscopy,  $400\times$ .