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Evaluation of crystallisation structures of cervical mucus in Zwartbles sheep with previous oestrus synchronisation

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Abstract: This study was conducted to determine the individual types of crystallisation structures of cervical mucus (arborisation phenomenon) in sheep and changes in the types of these structures during oestrus. A total of 80 ewes aged between two and eight years were included in the study. Oestrus synchronisation was performed using intravaginal sponges, and samples of cervical mucus were collected 55–57 h after the removal of intravaginal sponges and subsequently evaluated. The crystallisation structures of the cervical mucus of sheep exhibit the same sequence during oestrus as in cattle. The most frequently occurring types of crystallisation in the observed sheep included twig-shaped (33.33%), twig-shaped-clubmosses (24.00%) and clubmosses (14.67%). The least common type was fern frond crystallisation (1.33%). A statistically significant difference was found between the representation of individual types of crystallisation ($P < 0.01$). As with cattle, it is possible to use the evaluation of the crystallisation of cervical mucus to determine the optimum time for insemination, especially in groups of animals with previous synchronisation/induction of oestrus. Establishing the correct time for insemination or natural mating with a ram makes it possible to optimise breeding management and improve pregnancy rates, which are crucial in breeding these seasonally polyoestrous animals. Based on the results, it can be stated that insemination should take place 57+ h after the removal of intravaginal sponges and the application of hormonal treatments for oestrus induction and synchronisation (equine chorionic gonadotropin).

Keywords: arborization; detection; reproduction of sheep

Sheep are seasonally polyoestrous animals whose sexual activity is affected by the length of the day, and a reduction in daylight triggers the onset of the reproductive season (except in tropical regions, where reproductive activity is not affected by seasonal variations). The cycle of reproductive activity and rest can be divided into three basic periods: nonbreeding season, transitional period and breeding season (Romano 2021).

Oestrus synchronisation is employed in both dairy and meat breeds of sheep and serves primarily to enable the year-round production of animals and animal products. At the same time, it makes it possible to respond to rising price trends or take

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advantage of gaps in the market (Whitney and Jackson 2004).

Ewes with synchronised oestrus can be mated naturally with a ram (Maksimovic et al. 2020) or artificially inseminated (Bancheva et al. 2021). Oestrus synchronisation and artificial insemination (AI) are the most widely used ARTs (assisted reproductive technologies) in sheep breeding (Hameed et al. 2021). Artificial insemination can also be used in the reproduction of genetic reserves, for example, in Wallachian sheep in the Czech Republic (Savvulidi et al. 2021).

In the case of artificial insemination, establishing the ideal time for insemination is crucial to optimise pregnancy rates (Purdy et al. 2020). With ewes synchronised using intravaginal sponges, artificial vaginal insemination (with intracervical deposition of fresh diluted semen) should follow 56 ± 2 h after their removal (Madrigali et al. 2021).

One method of determining the optimal phase for insemination is evaluating the crystallisation of cervical mucus (Cortes et al. 2014), which has mainly been described in cattle breeding (Bernardi et al. 2016; Cortes and Vigil 2019). In the case of small ruminants, this method is less frequently employed, for example, in sheep and goats (Fonseca et al. 2017; Maddison et al. 2017). The crystallisation of other bodily fluids, such as saliva, can be used similarly (Skalova et al. 2013; Goncalves et al. 2020).

Cervical mucus performs several important functions: lubricating and moistening the epithelial surfaces of the female reproductive tract, acting as a natural sperm filter (Rutllant et al. 2005; Cortes et al. 2014), and a natural barrier preventing colonisation by unwanted micro-organisms and forming a cervical mucus plug in the cervix during pregnancy (Becher et al. 2009).

Cervical mucus is produced by mucus-secreting cells which are present in the cervical epithelium (Mullins and Saacke 1989). This cervical mucus is made up of 92–95% water (Tsiligianni et al. 2001). In addition, it contains soluble substances: proteins, enzymes, carbohydrates, amino acids, lipids (Rutllant et al. 2005; Siregan et al. 2019), and insoluble substances: electrolytes (primarily Na^+, K^+, Ca^{2+}, Cl^-), with the greatest influence on crystallisation coming from the content of salts: NaCl, KCl and $CaCl_2$ (Tsiligianni et al. 2001; Verma et al. 2014; Bernardi et al. 2016). The insoluble substances also include secreted gel-forming mucins, which have a major influence on the viscosity and elasticity of cervical mucus (Rutllant et al. 2005; Pluta et al. 2011; Reategui et al. 2017).

The composition of cervical mucus and its water content vary during the oestrus cycle. The main influence comes from the concentration of the hormones oestrogen and progesterone. Oestrogen (occurring principally in the period of oestrus) has a positive effect on the formation of cervical mucus and arborisation structures. Conversely, progesterone (in the luteal phase of the cycle or during pregnancy) posses a negative effect on the formation of cervical mucus (Skalova et al. 2013; Mandal et al. 2019). In addition to the creation of crystallisation structures and the water content of cervical mucus, oestrogen also has a positive effect on volume, flow elasticity/spinnbarkeit, cell content and protein content. Progesterone exerts a negative effect on these indicators (Linford 1974).

Crystallisation occurs when the water in cervical mucus evaporates, and some of its components tend to separate from the solution and precipitate in the form of crystals. Initially, molecular aggregation leads to the formation of crystal nuclei (nucleation), which subsequently increase in size and thus represent a precursor for the subsequent growth of individual crystals (Weber 1991; Skalova et al. 2013).

The formation of crystallisation structures of cervical mucus changes throughout the reproductive cycle. Some methodologies primarily concerned with evaluating the crystallisation forms of cervical mucus in cattle divide these crystallisation structures into several basic consecutive groups: twig-shaped and twig-shaped-clubmosses (pre-oestrus period), clubmosses (beginning of oestrus), clubmosses/fern frond (middle of oestrus) and fern frond (second half of oestrus) crystallisation. Other possibilities include swollen crystallisation (post-oestrus period), cellularisation (occurring during inflammation and metabolic disorders) or cervical mucus which does not form any crystallisation structures (non-oestrus period). The twig-shaped type is characterised by delicate structures that resemble twigs; the clubmosses type is characterised by branching structures that resemble plants of the genus Lycopodium; the fern frond type (also referred to as palm leaves) is characterised by a shape that resembles palm leaves or a fern frond. Swollen crystallisation produces enlarged branching and crumbling crystals. The cellularisation type crystallises into simple forms composed of several short segments (Cortes et al. 2014; Bernardi et al. 2016).

https://doi.org/10.17221/4/2023-CJAS

This study aimed to determine the individual crystallisation structures of sheep cervical mucus and subsequently to determine their sequence during oestrus in sheep.

MATERIAL AND METHODS

Animals and experimental design

The study was conducted according to the guidelines of the Declaration of Helsinki. Experimental procedures and animal care conditions followed the recommendation of European Union directive 86/609/EEC and were approved by Expert Commission for Ensuring the Welfare of Experimental Animals of Mendel University in Brno. Ethic Committee Name: The Ethics review board (The Ethics Committee of Expert Commission for Ensuring the Welfare of Experimental Animals) of Mendel University in Brno. Approval Code: 16OZ27083/2014-17214.

A total of 80 Zwartbles sheep were included in the experiment. Samples of cervical mucus were collected from 75 sheep to evaluate the crystallisation types. Five sheep served as a control group to determine the sequence of formation of crystallisation structures during oestrus and then compare the sequence of formation of these structures with the results reported in cattle.

The age of the sheep ranged from two to eight years. Animals with a similar BCS (body condition score) were deliberately selected for the experiment. The average BCS value was 3 (min. 2.5, max. 3.5).

Preparation of the animals, synchronisation of oestrus (using intravaginal sponges) and subsequent collecting of samples of cervical mucus from the ewes was carried out from September to October 2021. A total of 75 samples of cervical mucus were collected to determine the types of crystallisations of cervical mucus in the primary group, and 20 samples (four sample collections from five animals) of cervical mucus were taken from the control group. All of the samples collected were used in the study.

Synchronisation of oestrus

Before the actual synchronisation of oestrus, flushing was employed to modify the physical condition of the ewes and promote oestrus symptoms. Oestrus synchronisation was carried out from late September to early October 2021. The actual synchronisation was performed using Ovigest intravaginal sponges (medroxyprogesterone acetate 60 mg; Laboratorios Hipra s.a., Amer, Spain). The sponges were inserted into the vagina for 14 days using a plastic applicator.

After their removal, the lyophilised serum gonadotropin Sergon (equine chorionic gonadotropin 500 IU/ml; Bioveta a.s., Ivanovice na Hané, Czech Republic) was applied intramuscularly to the ewes at a dosage of 200 IU/sheep to promote oestrus synchronisation, oestrus symptoms and subsequent conception.

Collection of cervical mucus samples of the primary group

A total of 75 samples were collected from the 75 sheep included in the study. These samples were subsequently used to determine the individual types of crystallisation structures.

The samples of cervical mucus were collected 54 to 58 h after the removal of the intravaginal sponges and application of Sergon. The mucus samples were collected from the vaginal region of the individual ewes (in front of the cervix) with a sterile plastic pipette through a vaginal speculum in order to prevent contamination with impurities from the external sexual organs. After each sample collection, the plastic pipette was replaced, and the vaginal speculum was disinfected.

Immediately after collection, each sample was placed on a marked (number of ewe) microscope slide and smeared at an angle of 45°. The samples were allowed to dry at room temperature (cca 22 °C, for 5 to 7 min until completely dry). After drying, the samples were stored in marked plastic folders until the time of the evaluation.

Collection of cervical mucus samples of the control group

A total of 20 samples were taken from five animals. These samples were subsequently used to verify the sequence of formation of crystallisation structures during oestrus and then compare the sequence of formation of these structures with the results reported in cattle.

https://doi.org/10.17221/4/2023-CJAS

Samples were collected and stored in the same way as with the primary group. A sample of cervical mucus was taken from each ewe in the control group 48, 51, 54 and 57 h after the removal of the intravaginal sponge and application of Sergon.

Evaluation of cervical mucus samples

Cervical mucus smears were subsequently examined on the day of collection and observed under a microscope (Olympus BX51TF; Olympus Corporation, Tokyo, Japan) at 200× magnification and the type of crystallisation was recorded.

The procedure for creating smears, their evaluation and the types of crystallisation structures were determined by standard methodologies for assessing the crystallisation of cervical mucus in cattle. Samples were subsequently divided into nine groups according to the crystallisation type: type N (without crystallisation), type N + T (weak twig-shaped crystallisation), type T (twig-shaped), type T + C (twig-shaped-clubmosses), type C (clubmosses), type C + F (clubmosses/fern frond), type F (fern frond), type S (swollen crystallisation) and type CE (cellularisation).

Examples of the individual types of crystallisation are given in Figure 1.

Statistical analysis

The software used to perform the statistical analysis was Statistica 14 (StatSoft CR s.r.o., Prague, Czech Republic). Data were analysed using Chi-square test analysis. The frequency difference for individual types of crystallisation structures was tested (Table 1).

RESULTS

Observed types of crystallisation

In the first part of the experiment, a total of 75 samples taken from 75 animals were evaluated. The representation of the individual types of crystallisation is shown in Table 1 and Figure 2. The most common types of crystallisation were twig-shaped (33.33%), twig-shaped-clubmosses (24.00%) and clubmosses (14.67%), which occurred in a total of 72.00% of the observed animals. Less common types of crystallisation were clubmosses/fern frond (8.00%), weak twig-shaped crystallisation (5.33%) and cellularisation (5.33%). The least common were swollen crystallisation (4.00%), samples without crystallisation structures (4.00%) and fern frond crystallisation (1.33%).

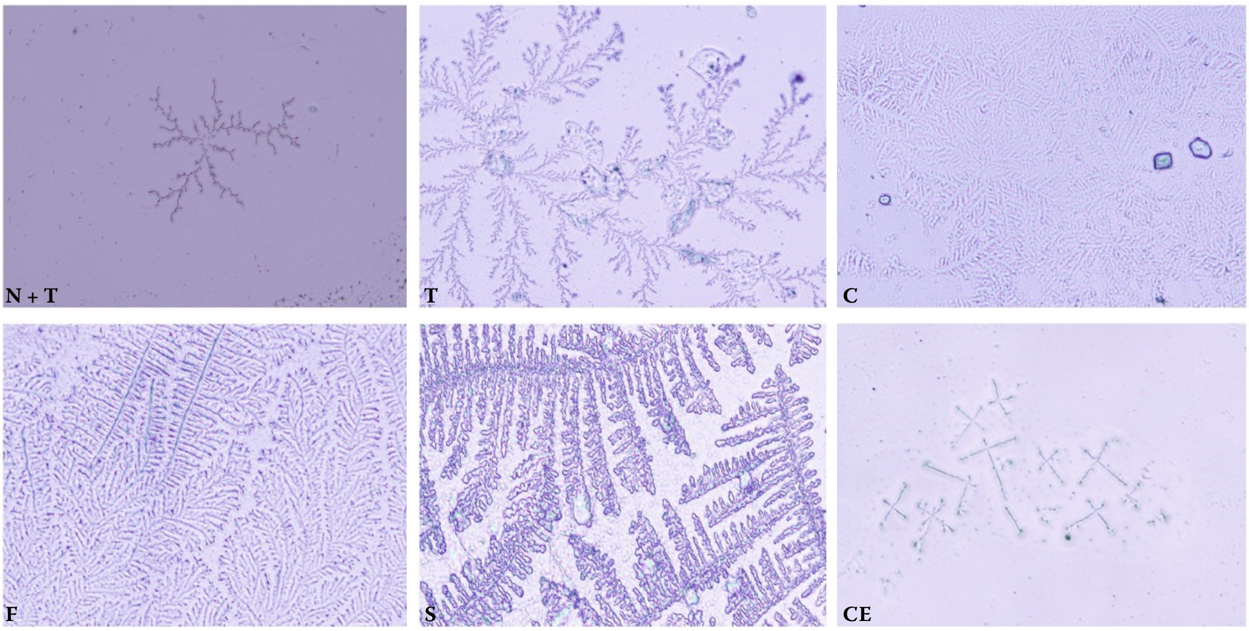

Figure 1. Examples of crystallisation types (original examples from our research)
C = clubmosses; CE = cellularisation; F = fern frond; N + T = weak twigg-shaped crystallisation; S = swollen; T = twigg-shaped

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Table 1. Crystallisation types of cervical mucus (n = 75)

Type of crystallisation	Mark	Samples (n)	x (%)	(O–E)^2	P-value (χ^2 test)	SD	CV
Without crystallisation	N	3	4.00	4.551 1			
Weak twigg-shaped	N + T	4	5.33	3.004 4			
Twigg-shaped	T	25	33.33	44.444 4			
Twigg-shaped-clubmosses	T + C	18	24.00	14.951 1			
Clubmosses	C	11	14.67	1.137 8	0.000 1	10.873 0	0.978 6
Clubmosses-fern frond	C + F	6	8.00	0.871 1			
Fern frond	F	1	1.33	8.604 4			
Swollen	S	3	4.00	4.551 1			
Cellularisation	CE	4	5.33	3.004 4			

(O–E)^2 = χ^2 test (63.840 0), df = 8, P < 0.01; CV = coefficient of variation; SD = standard deviation; x = percentage representation

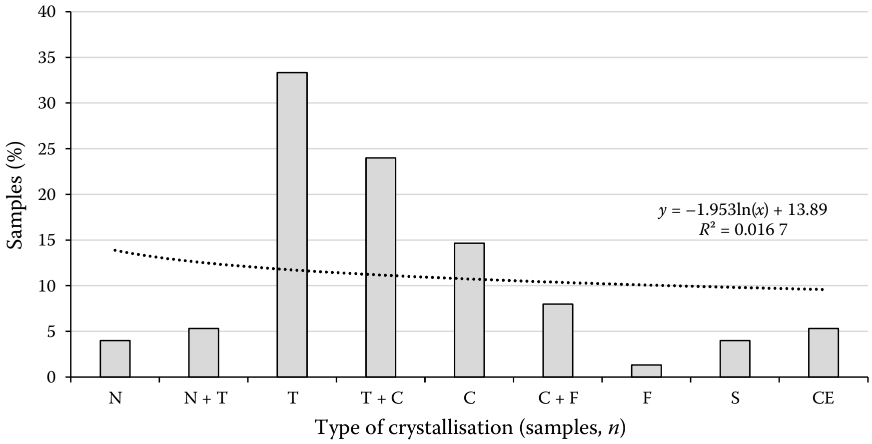

Figure 2. Total representation of types of crystallisation structures of cervical mucus
C = clubmosses; C + F = clubmosses/fern frond; CE = cellularisation; F = fern frond; N = without crystallisation; N + T = weak twig-shaped crystallisation; S = swollen crystallisation; T = twig-shaped; T + C = twig-shaped-clubmosses

A highly statistically significant difference was found between the representation of individual types of crystallisation structures (Chi-square test 63.840 0, P = 0.000 1, Table 1).

The sequence of crystallisation types

During observation changes occurred in the types of crystallisation forms, which are shown in Figure 3. On first observation (48 h after Sergon application) there were isolated examples of swig-shaped structures (N + T) in 40.00% of the observed animals, while in 60.00% crystallisation structures had not yet formed (N). By the 51st hour, twig-shaped crystallisation (T) had occurred in all of the observed animals. By the 54th hour type T crystallisation had occurred in 60.00% and type T + C crystallisation in 40.00% of the animals. 57 h after applying Sergon, there were type

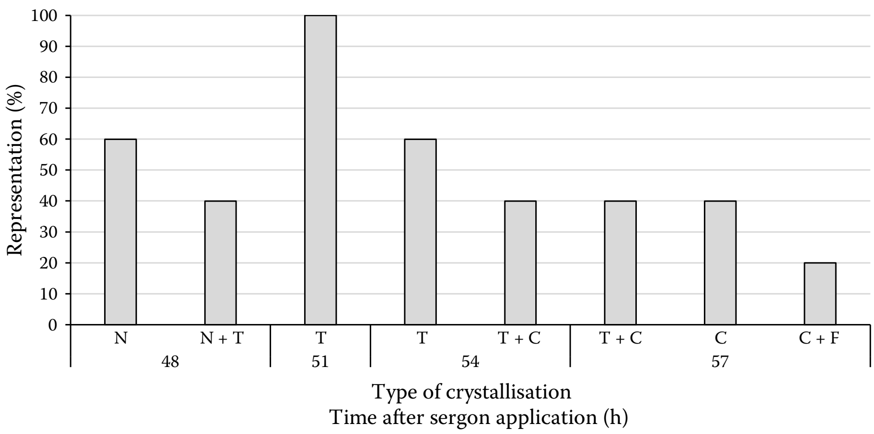

Figure 3. Sequence of crystallisation types during oestrus
C = clubmosses; C + F = clubmosses/fern frond; N = without crystallisation; N + T = weak twig-shaped crystallisation; T = twig-shaped; T + C = twig-shaped-clubmosses

https://doi.org/10.17221/4/2023-CJAS

T + C crystallisation structures in 40.00%, type C in 40.00% and type C + F crystallisation in 20.00% of the animals.

DISCUSSION

During oestrus, there are changes in the types of crystallisation of cervical mucus, which depend primarily on the concentration of the hormones oestrogen and progesterone (Skalova et al. 2013; Mandal et al. 2019). The sequence of types of these crystallisation structures observed in the control group of sheep exhibited the same variability as in studies dealing with the arborisation phenomenon in cattle (Cortes et al. 2014).

The samples collected 48 h after Sergon application contained isolated examples of twig-shaped crystallisation, or else crystallisation was not present in them at all. This type of crystallisation occurs primarily in the period prior to oestrus and at the beginning of oestrus. Due to the low level of oestrogen during pregnancy, crystallisation structures do not form in this period either (Cortes et al. 2014). The crystallisation forms subsequently increased in size and changed from the twig-shaped type (51–54 h after Sergon application) to twig-shaped-clubmosses (54–57 h after Sergon application) and clubmosses/fern frond (57 h after Sergon application). It can be assumed that if the observation period was extended to more than 57 h, this would lead to a greater occurrence of fern frond crystallisation, which is formed in the second half of oestrus (Cortes et al. 2014).

In 72.00% of the observed animals, the crystallisation forms T, T + C, or in some cases, C, occurred 54–58 h after Sergon application. These are forms which occur during oestrus, especially in the first half of it (Cortes et al. 2014). The minimal representation of subsequent types of crystallisation (C + F, F) was probably due to the samples of cervical mucus having been taken during the earlier phase of the oestrus cycle when these types of crystallisation had not yet formed (Jezkova et al. 2008; Bernardi et al. 2016).

The occurrence of cellularisation of the crystallisation structures indicates the presence of inflammation. Swollen crystallisation has a structure with a straight main axis and ramifications protruding at an angle of 90°, and it is a characteristic type of crystallisation occurring in the post-oestrus period (Jezkova et al. 2008; Cortes et al. 2014).

Determining the type of crystallisation makes it possible to establish the optimal time for insemination (Mandal et al. 2019), which in cattle should be carried out when the crystallisation types C + F and F are present (Bernardi et al. 2016). The type of crystallisation also affects the ability of sperm to survive in the female reproductive tract (Jezkova et al. 2008). The properties, quality and types of crystallisation structures of cervical mucus can differ between animals with natural and induced onset of oestrus (Tsiligianni et al. 2001). The presence of some non-naturally occurring types of crystallisation can also make it possible to detect health problems (cellularisation = presence of inflammation, atypical crystallisation = metabolic disorders) (Jezkova et al. 2008; Cortes et al. 2014).

The ability of sperm to survive in cervical mucus is closely linked to conception rates. The lowest rates of sperm survival and subsequent conception are associated with insemination in the period when crystallisation structures are absent from cervical mucus or atypical crystallisation structures are formed. Conversely, the best rates of sperm survival and conception are achieved when insemination is carried out at the time when clubmosses and fern frond crystallisation are formed (Jezkova et al. 2008).

Evaluating the crystallisation of cervical mucus can thus be used as a simple method of detecting oestrus but can also be used to diagnose gravidity. To facilitate the process, it is also possible to use samples of saliva or other bodily fluids (nasal mucus, tears, milk/colostrum) instead of cervical mucus (Skalova et al. 2013).

CONCLUSION

Given that the sequence of formation of crystallisation structures of cervical mucus in sheep is the same as the results shown in cattle, the search for the optimal phase for insemination/mating can also be similarly applied to sheep, with the difference that in the case of insemination/mating in sheep based on crystallisation structures, further research is necessary to evaluate the effect of the type of cervical mucus crystallisation on conception rates success. The possibility of quick-

https://doi.org/10.17221/4/2023-CJAS

ly determining crystallisation types, for example by using portable pocket microscopes (e.g. FerTest, MEOPTA a.s., Přerov, Czech Republic), allows for the optimisation of the insemination/mating time, thus improving subsequent conception rates.

Conflict of interest

The authors declare no conflict of interest.

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