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Saliva crystallization in cattle

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Chief supervisor: Ing. Karolína Koláčková, PhD.

Second (specialist) supervisor: Ing. Tamara Haberová

Author: Iva Skálová

Statement

I declare that I worked out this M.Sc. diploma thesis titled „Saliva crystallization in cattle“ alone and that I used only literature listed in references.

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Iva Skálová

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Author's Abstract

Saliva crystallization in cattle

The accurate heat and pregnancy detection is one of the decisive factors of successful livestock breeding. Available methods in management of livestock reproduction are time-consuming and expensive. The saliva sampling is simple and noninvasive procedure, which is applicable in untamed animals and the presence of veterinary is not needed. The phenomenon of crystallization of body fluid is known over 60 years. The saliva crystallization was mainly described in women. Only two studies focused on saliva crystallization in animals are available. The aim of this thesis was confirmation of presence of saliva arborization in cattle and observation of changes in crystallization during synchronized cycle and pregnancy. The Giemsa stain was additionally tested as tool for better searching of crystals. The eight cows of Holstein breed were included into research and 408 samples were microscopically evaluated in total. The samples were obtained daily from the first application of preparation of synchronization till 34th day after insemination. The crystals were scored according to following system: 0 = none, D = dotted, BL = branch-like, FIL = fir-like, FEL = fern-like, BL+FIL = mixed branch-like and fir-like, BL+FEL = mixed branch-like and fern-like, FIL+FEL = mixed fir-like and fern-like, BL+FIL+FEL = mixed branch-like, fir-like and fern-like, A = atypical. All mentioned types of crystallization were confirmed in tested animals as well as the changes during whole observed period. At the time of artificial insemination were presented only two types of arborization (BL+FIL and BL+FIL+FEL), but these types are on lower level of development. The predominant types of crystallization, noticeable after insemination (FIL+FEL, FIL, BL), were marked in only in pregnant animals. The changes in dominance of these types varied with increasing number of days after insemination. It was confirmed, that sample quality influenced the density of crystals. Some of crystalline patterns, detected in cattle saliva, were similar with crystals described in other studies focused on crystallization of body fluids.

Key words: reproductive cycle, crystalline patterns, arborization, ferning, heat and pregnancy detection

Autorský referát

Krystalizace slin skotu

Správná detekce říje a březosti je jedním z rozhodujících faktorů úspěšného chovu hospodářských zvířat. Dostupné metody v managementu reprodukce hospodářských zvířat jsou časově i finančně náročné. Odběr vzorků slin je jednoduchý a neinvazivní zákrok, který lze aplikovat i u téměř neochočených zvířat a k jeho realizaci není zapotřebí asistence veterinárního lékaře. Jev krystalizace tělních tekutin je znám již přes 60 let. Krystalizace slin byla více popsána u žen. Existují pouze dvě studie zabývající se touto krystalizací u zvířat. Cílem této práce bylo potvrdit přítomnost krystalizace ve slinách domácího skotu a sledovat její změny během synchronizace říje a březosti. Dále bylo testováno barvivo Giemsa, jako pomůcka pro lepší hledání krystalů. Do studie bylo zařazeno 8 krav holštýnského plemene a mikroskopicky bylo vyhodnoceno celkem 408 vzorků. Vzorky byly odebírány denně od první aplikace synchronu do 34. dne po inseminaci. Krystaly byly klasifikovány podle desetistupňového systému: 0 = žádná, D = tečkovitá, BL = větvičkovitá, FIL = jedlovitá, FEL = kapraďovitá, BL+FIL = větvičko-jedlovitá, BL+FEL = větvičko-kapraďovitá, FIL+FEL = jedlovito-kapraďovitá, BL+FIL+FEL = větvičko-jedlovito-kapraďovitá, A = atypická. Všechny typy krystalizace byly potvrzeny u testovaných zvířat, stejně tak jako její změny během celého sledovaného období. V den inseminace dominovaly pouze dva typy krystalizace (BL+FIL, BL+FIL+FEL), které ale nebyly nejvyššího stupně vývoje. Po inseminaci se převládající typy krystalizace (FIL+FEL, FIL, BL) vyskytovaly jen u březích zvířat a změna dominance jednotlivých typů se měnila s přibývajícími dny po inseminaci. Potvrdilo se, že kvalita vzorku ovlivnila hustotu krystalů ve vzorku. Některé krystalické útvary se shodovaly s krystaly popsaných v jiných studiích.

Klíčová slova: reprodukční cyklus, krystalické útvary, arborizace, detekce říje a březosti, cervikální hlen

List of tables:

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List of the contractions used in this thesis:

A - atypical

ACTH – adrenocorticotrophic hormone

BL – branch-like

BL + FIL + FEL - mixed branch-like and fir-like and fern-like

BL+FEL – mixed branch-like and fern-like

BL+FIL mixed branch-like and fir-like

bPSPB – bovine Pregnancy Specific Protein B

CL – corpus luteum

CLs – corpora lutea

CMIr – ratio of chloride to ″residual″ protein

CMIt - ratio of chloride to total protein

CULS – Czech University of Life Science Prague

D – dotted

DOF – degrees of freedom

EPF – early pregnancy factor

FEL – fern-like

FIL – fir-like

FIL + FEL – mixed fir-like and fern-like

FSH – follicle stimulating hormone

GnRH – gonadotropin - releasing hormone

K – potassium

KCl – potassium chloride

LH – luteinizing hormone

Na – sodium

NaCl – sodium chloride

O – none

PGE_2 - prostaglandin E_2

$PGF_{2\alpha}$ - prostaglandin $F_{2\alpha}$

PIF – prolactin - inhibiting factor

Content

1. Introduction

Heat and pregnancy detection is one of the decisive factors in sustainable breeding of dairy cows. The reproductive success rate depends on healthy females, heat detection, timing the insemination, using the high quality semen and proper insemination technique. The insemination should be timed in correlation to estrus, because ovulation is very difficult to detect (Gündoğan, 2008).

A lot of techniques for a detection of a heat and pregnancy are quite expensive, demanding for a time and some methods must be done by a veterinary. A obtaining of saliva samples is very simple process, which can be performed by a breeder.

The phenomenon of crystallization was studied in numerous researches. The crystallization of human cervical mucus was discovered by Papanicolaou in 1946, the relationship between crystalline patterns and an estrogen activity was noticed in this study. In 1951, Campoz da Paz reported the inhibiting influence of progesterone on crystallization (in Forman, 1956).

The crystallization of cervical mucus or the other body fluids was assessed in many species, such as cows (Alliston et al., 1958; Noonan et al.,1975; Ahmadi et al., 2005), rhesus monkeys (David & Mastroianni, 1968), pigs (Haynes, 1970; Betteridge & Raeside, 1962), ewe (Raeside & McDonald, 1959) and bitches (England & Allen, 1989). The crystallization occurs also in the other body fluids, such as in nasal mucus (Peterson, 1984), saliva (Pardo-Carmona et al., 2010), cerebrospinal fluid, fluid from follicles, hydrosalpinx and ovarian cysts (Zondek, 1954; in Golding & Brennan, 1989).

Saliva crystallization was mostly described in women (Kullander & Sonesson, 1965; Barbato et al., 1993; Guida et al., 1993; Berardono et al., 1993; Pattansuttinont et al., 2007). The description of saliva crystallization in animals was presented by Pardo-Carmona et al. (2010), who tested crystals in saliva for determining optimal mating time in bitches. Other study of animal saliva was mentioned by Haberová (2010), where changes in the saliva crystallization of camels (*Camelus bactrianus*) were observed. The saliva arborization in cattle has not been described yet.

2. Aims of the Thesis

The main goal of this thesis was the verification of the presence of saliva crystallization in domestic cattle during reproductive cycle and pregnancy.

After confirming the creation of crystals, it was necessary to find the connection between the type of crystallization and phase of the reproductive cycle or the pregnancy of tested animals.

The other aim was to try Giemsa stain as a tool for better searching of crystals in cattle saliva.

Further goal of this study was the evaluation of applicability of saliva crystallization in practice for monitoring of the reproductive cycle and pregnancy diagnosis in cattle.

The phenomenon of saliva crystallization was described from available resources in the bibliographic research and compared with other known methods connected with crystallization.

2.1. Hypotheses

1. The saliva crystallization will appear in cattle.
2. The saliva crystallization will match up with the crystallization of cervical mucus in cattle.
3. The saliva crystallization in cattle will correspond to reproductive cycle.
4. It should be possible to determine phase of reproductive cycle according to the type of crystallization: the type will be influenced by the phase of reproductive cycle.
5. The type of crystallization will differ in pregnant and cycling animals.

3. Bibliographic Research

3.1. Cattle reproduction

The breeding of cattle is the main sector of animal husbandry in Europe. According to the Czech Statistical Office in Czech Republic, the number of cattle is 1, 350, 000 pieces of cattle, of which 380, 000 are dairy cows and 170, 000 are cows used for calves production. Countries with the most important dairy cattle breeding programs are France, Germany, Great Britain and Poland. The economics of cattle husbandry is influenced by fertility of cows. In females the fertility is evaluated by production of gametes, oocyte fertilization, maintenance and finishing of pregnancy, optimal parturition and number of viable calves (Stupka et al., 2010).

The maturation in cows is connected with production of gametes (Stupka et al., 2010). In heifers this secretion occurs in age of 9 months. The age of puberty in cows is in range 7-18 months (Noakes et al., 2001). In Holstein cows the first mating is around 13-14 months of age (Stupka et al., 2010).

The reproduction in cattle is directed by the nervous system and hormones. The basis of this system is the cascade hypothalamus – pituitary gland – gonads. The cows belong to polyestrous animals, thus they can go into heat several times a year which can be repeated in periodic cycles (Bouška et al., 2006).

The length of cycle is usually 21 days. Typical estrus behavior occurs immediately before ovulation. The last sign of estrus is acceptation of a bull. The cycle is divided into four stages (Peters & Ball, 1986). The pre-estrus phase is called proestrus which lasts three days, cycling on the 19^{th}, 20^{th} and 21^{st} day. In this period new follicle is created. The estrus, second phase, lasts 12-36 hours and includes 1^{st} and 2^{nd} day of cycle. This time is represented by Graaf´s follicle maturation, cervix opening, flowing of mucus from vulva. In 6-16 hours after subsiding of estrus signs the ovulation comes. Metestrus ensues after estrus which lasts commonly 4 days. On ovary the corpus luteum is developed. The corpus luteum becomes larger in diestrus, which lasts usually 12 days. In the case of unsuccessful insemination the corpus luteum begins to regress. On the other hand in pregnant animals diestrus proceeds to gestation (Marvan et al., 2007).

The pregnancy lasts 285-290 days. The parturition has three stages: preparatory phase, expulsion of the fetus and expulsion of fetal membranes. Usually cow has one

calve. Multiple births are occurred in 1-4%. The lactation begins after parturition and is hormonally regulated. The normalized lactation is determined in 305 days. The new periodic sexual functions start around 42nd day after calving. The first heat after parturition is usually silent. Under normal conditions it is possible to do insemination in 60 after birth. The optimal length of calving interval is 365-370 days, this means one calve from cow per year (Stupka et al., 2010).

3.1.1. Heat and pregnancy diagnosis

Successful heat detection is a main aid in determination of optimal time for artificial insemination. Detector animals, tail head markings, pressure-sensitive mount detectors, ultrasonography, electrical resistance of reproductive tract, ultrasonography, pedometers and electronic pressure sensitive mount detectors belong among means used for heat detection (Hafez & Hafez, 2000).

Requirements for visual heat detection include identifying multiple signs of estrus from signs of forthcoming estrus, time exploitable only for monitoring of all cows, frequency of observation per day (Hall et al. 1959).

Visual observation detects estrus behavior. The changes in expression during estrus are described as restless, nervousness, mounting, noisy, increase degree of walking, sniffing, nudging, putting the tail to the side during contact with other animal, swelling of vulva, clear mucus from vulva, low appetite and increased interest in surroundings (Foote, 1974).

The detectors of mounting are placed in front of the beginning of tail. The instrument is activated by animal mounting. Marking the tail head is based on the same principle (Hafez & Hafez, 2000). In the study of Stevenson and Britt (1977) cows were observed by rump-mounted detector. The results of measurements were supplemented by serum progesterone.

The electronic pressure-sensitive mount detector, radiotelemetric estrus detection, works on the base of sending radiotelemetric signal. The sensor is located in the midsacral region of the cow and a signal is transmitted over antenna to software program (Dransfield et al., 1998).

For heat detection by animals are used vasectomized or surgically altered bulls and hormone treated cows (Hafez & Hafez, 2000). The method with androgenized animals was also used in the study of Stevenson and Britt (1977).

The decreasing electrical resistance was described in vaginal fluids during proestrus and estrus (Hafez & Hafez, 2000). Lewis et al. (1989) used for experiment radiotelemetric sensor, which was introduced through the vulva to measure electrical resistance of tissues.

Due to higher activity of cows during estrus, many breeders use pedometers. The counter is placed on leg or neck (Hafez & Hafez, 2000). Maatje et al. (1996) used pedometers with an alarm signal. The heat detection by pedometers is based on the comparing activity between two periods.

All of modern electronic technologies could be used for enhancing of heat detection. On the other hand the pedometers should be supplemented with other method for heat detection, usually visual observation (Rorie et al., 2002).

The steps which can improve effectiveness were reported in Hafez and Hafez (2000) as: better identification of animals, promotion and observation of cow relationships, the best nutrition and health, good footing surface, usage of accurate records and devices, determination of employee responsibility, good measures and hormonal synchronization programs.

The estrus synchronization helps to timing of artificial insemination. Due to hormonal regulated estrus the accurate time of ovulation could be expected (Hafez & Hafez, 2000). Nowadays the method of estrus synchronization is usually used in modern livestock husbandry.

The correct decision about gestation is one of the main tools of suitable management of reproduction. One method how to evaluate herd fertility is the successful designation of pregnancy.

The pregnancy diagnosis includes observation and also physical examination, tests on chemical base and electronic instruments (Hafez & Hafez, 2000).

The intention in pregnancy determination in bovine is not to detect those that are pregnant but recognize those that are not pregnant so that they can be reinseminated or removed from the herd. The ideal age for first calving in dairy cows is 24 months (Hafez & Hafez, 2000).

A lot of methods for diagnosis of pregnancy are effective between 25 and 40 days after conception. There is no test which is usable in pregnancy diagnosis before the first expected estrus (Hafez & Hafez, 2000).

The onset and maintenance of pregnancy in most domestic species is caused by prolonged luteal phase of the estrus and by the presence of a single corpus luteum (CL) or a number of corpora lutea (CLs) (Noakes et al., 2001).

Among management methods for pregnancy is arranged exposure to a bull or artificial insemination, failure to return estrus or cessation of the estrus cycle, presence of corpus luteum, metesrus bleeding, visible changes in mammary glands and abdominal ballottement (Hafez & Hafez, 2000; Noakes et al., 2001)

Many owners consider the coexistence of cow and bull as a sufficient indicator for pregnancy detection. Cows that became pregnant refuse the bull and its efforts to mating. Non return estrus is caused by the presence of embryo around 15 – 17 days after ovulation. In this period the corpus luteum is maintained and the estrus cycle is arrested. Successful conception is characterized by cessation of the estrus cycle. Also metestrus bleeding could be used for recognizing a non pregnant cow. If this bleeding is monitored in a cow that was no detected in estrus a few days previously, it is an indication that estrus was unrecorded and that this animal is not pregnant (Hafez & Hafez, 2000).

The changes in mammary glands are best visible in cows pregnant for the first time. During the fourth months the teats start to enlarge and so it is easy to differentiate non – pregnant and early pregnant heifers. The mammary gland becomes harder from sixth month. The biggest enlargement and oedematous tendency are observed during the onset of parturition (Noakes et al., 2001).

Abdominal ballottement is the last management method. It is based on quite spirited banging on the ventral part of abdomen. The mean of this operation is to press the fetus (Noakes et al., 2001).

Other way how to investigate cows for pregnancy is palpation per rectum. This method is practicable around day 30 after conception and then until parturition. Four indications of pregnancy are determined by rectal palpation: fetal membrane slip (allantochorion), amniotic vesicle, placentomes and presence of fetus (Hafez & Hafez, 2000).

Palpation of caruncles (cotyledons), which are created on the gravid horn, is double at 10-11 weeks. From about 3 months cotyledons looks like structures in the midline. During gestation the caruncles become bigger (Noakes et al., 2001).

Difference in size of uterine horns, flexibility and fluctuance of the uterine wall, fixation of the cervix, hypertrophy of the uterine artery, and changes on the ovaries belong among auxiliary signs of positive pregnancy (Hafez & Hafez, 2000).

Various methods including chemical tests, usually called laboratory techniques, are also used for pregnancy diagnosis. The progesterone test is based on determination the level of progesterone in blood and milk. The samples of plasma or milk are taken around day 21 or even earlier after the previous estrus. In cases of low degrees of progesterone in blood or in milk, the cow is considered to be non-pregnant (Peters & Ball, 1986; Noakes et al., 2001).

An early pregnancy factor (EPF) is an immunosuppressive glycoprotein connected with gestation. This method is still experimental phase (Hafez & Hafez, 2000).

The level of bovine Pregnancy Specific Protein B is measurable between day 15 and 24 after insemination. During pregnancy levels of bPSPB increase and is ascertained until calving (Hafez & Hafez, 2000).

The placenta produces estrone sulphate (Hoffmann & Schuler, 2002; Eley et al., 1979). It is detectable in milk in pregnant and lactating animals, while in non-pregnant animals low concentration are noticed or estrone sulphate is untraceable (Hafez & Hafez, 2000; Noakes et al., 2001).

Vaginal investigation can be done manually or visually. For examination an illuminated speculum is employed (Noakes et al., 2001).

From 6-7 weeks of pregnancy, the ultrasonographic method should be used. Examination of early pregnancy is based on non-echogenic area (black) and on the other hand the reaction on the presence of fetal fluids. The confirmation of pregnancy is conditioned by the embryo or fetus detection (Noakes et al., 2001).

For multiple gestations determination fetal electrocardiography is used (Noakes et al., 2001).

3.1.2. Hormones and hormonal changes

The reproduction of mammals is controlled by central nervous system and endocrine systems. The hormones are defined as agents on a chemical, organic or

physiological basi and have inhibitory, stimulatory and regulatory effects on organs and tissues. The hormone classification is according to type of action or biochemical structure. On the basis of biochemical structure hormones are categorized to four groups: proteins, steroids, fatty acids and amines (Hafez & Hafez, 2000).

The reproductive processes are directed by primary hormones while reproduction is regulated by metabolic or secondary hormones. The hormones of cattle reproduction are originated from hypothalamus, pituitary gland, gonads, uterus or placenta (Hafez & Hafez, 2000).

Among hypothalamic releasing hormones belong gonadotropin-releasing hormone (GnRH), prolactin – inhibiting factor (PIF) and adrenocorticotrophic hormone (ACTH). Oxytocin and vasopressin are also released in hypothalamus and they are maintained in neurohypophysis. The surges of GnRH activate secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Hafez & Hafez, 2000).

From adenohypophysis FSH, LH and prolactin are released, which are counted into gonadotropic hormones (Hafez & Hafez, 2000). The main role of FSH is instigation of follicles to grow and maturate (Reece, 1998). The preovulatory pulses of LH cause the rupturing of a follicle and consequently ovulation. The creating of corpus luteum is other function of LH (Reece, 1998). The FSH and LH are under estrogens and progesterone control**.** During higher concentration of estrogen secretion of LH and FSH decreases. Contrary in case of increased influence of progesterone the releasing effect of gonadotropins is inhibited (Reece, 1998). In study of Dobson et al. (2000) cows were treated by ACTH and LH profiles were decreased, but FSH levels were similar. After parturition levels of FSH increase and ensues determination of next dominant follicle (Crowe et al., 1998; Beam & Butler, 1997). A few follicular waves are typical for estrus cycle in bovine, usually two or three (Savio et al., 1991; Savio et al., 1990). The prolactin inhibiting factor (PIF) provides prolactin production. This hormone is responsible for lactation (Hafez & Hafez, 2000).

Neurohypophyseal hormones are only stored in neurohypophysis. Oxytocin and vasopressin are transferred by nervous system from hypothalamus to posterior pituitary. The second source of oxytocin is corpus luteum in ovaries. Oxytocin influences contractions of uterus during estrus and parturition. Suckling or milking increase the production of oxytocin. Oxytocin from ovaries stimulates secretion of prostaglandin $F_{2\alpha}$

($PGF_{2\alpha}$) which caused luteolysis. Melatonin produced in pineal gland is responsible for seasonality in small ruminants (Hafez & Hafez, 2000).

$PGF_{2\alpha}$ belongs to prostaglandins together with prostaglandin E_2 (PGE_2). $PGF_{2\alpha}$ activates new reproductive cycle after unsuccessful fertilization (Hafez & Hafez, 2000). The prostaglandin $F_{2\alpha}$ is used in estrus synchronization (Rathbone et al., 2001).

The prostaglandins are considered as agents of contractions of muscles in reproductive and gastrointestinal tracts, creation of corpus luteum, ovulation, calving and expulsion of milk (Hafez & Hafez, 2000).

The gonadal steroid hormones affect sexual behavior in mammals (Ford & D´Occhio, 1989). The hormones of gonads in females are classified into steroids (estrogens, progestins) and protein (relaxin). Two main types of steroid are produced by ovaries: 18-carbon estrogens and 21-carbon progestins (Hafez & Hafez, 2000). The estrogens occur in natural or synthetic form (Reece, 1998). All ovarian estrogens are substituted by estradiol (primary estrogen), estriol and estrone (Hafez & Hafez, 2000). The 17ß estradiol dominates in non-pregnant animals and on the other hand the estrone prevails in pregnant animals, where the volume of estrone increases rapidly 3-4 weeks before parturition (Reece, 1998). The estrogens are responsible for development of secondary reproductive signs, mammary gland and duct (Hafez & Hafez, 2000). Also the estrogens evoke sexual behavior (Reece, 1998). In ruminants the estrogens are connected with gains in body weight. Progesterone is produced by cells of corpus luteum, placenta and adrenal gland (Hafez & Hafez, 2000). In ACTH treated animals levels of progesterone decreased (Dobson et al., 2000). The activities of progesterone are usually connected with estrogen action (Reece, 1998). The main function of progesterone is to maintain the pregnancy and help to develop alveoli in mammary gland. The progesterone inhibits uterine contractions during gravidity (Reece, 1998). For estrus synchronization is used synthetic progesterone (Hafez & Hafez, 2000).

With pregnancy is also connected hormone relaxin (Hafez & Hafez, 2000). In mammals, relaxin achieves highest value during gestation (Anderson et al., 1982). It is produced mainly by corpus luteum and in some species also by uterus and placenta. The main role of relaxin is expansion of cervix and vagina during onset of parturition (Hafez & Hafez, 2000). According to the results of Musah et al. (1986) cows treated with relaxin had shorter pregnancy and the cervix expanded to the maximum during a few hours after treatment. The stimulatory influence of treated relaxin on cervix dilatation, pelvic

expansion, progesterone decreasing and sooner calving was confirmed by Badinga et al. (1992).

The inhibins and activins, from gonadal fluids, have influence on regulation of FSH and LH. The inhibins control hormonal regulation of follicle development during reproductive cycle. The inhibitory influence on FSH production has follistatin, which is also isolated from follicular fluid (Hafez & Hafez, 2000).

The group of placental hormones in cows includes placental lactogen, which is important for fetus nutrition and its growth. In dairy cows it was noticed that there were higher amounts of placental lactogen than in beef cows (Hafez & Hafez, 2000).

For pregnancy diagnosis is used protein B, which preventively influences regression of corpus luteum during early pregnancy (Hafez & Hafez, 2000).

3.2. Crystallization

During the period of increased level of estrogens in the blood many body fluids, mainly cervical mucus, nasal mucus and saliva, created specific crystalline patterns after they were smeared and left dry on the glass slide. The changes of these crystals are influenced by increasing or decreasing level of estrogens. In the luteal phase of the reproductive cycle the crystallization expires because of progesterone causing (Rob & Stehlík, 1983).

Physiologically the crystals are created during the proestrus and the heat. From the pathological point of view the crystallization is visible with increased intake of the feeds with fytoestrogens or feeds contaminated by fungus genus *Fusarium* (Rob & Stehlík, 1983).

The crystallization phenomenon has been known over sixty years (Papanicolaou, 1946; Rydberg, 1948). Yamane (1921) observed the crystals in semen from cattle, horses and rabbits (in Kihlström & Fjellström, 1969). In 1946, Papanicolaou described, that the cervical mucus created the crystals, which were represented a kind of crystallization. This mucus was smeared on the glass slide and left dry. It was noticed, that these crystals were distinctive near the peak of follicular activity and around ovulation time. During the other phases of reproductive cycle the reduction or absence of crystallization were observed (MacDonald, 1969).

Rydberg (1948) described the crystallization as resembling fern or palm leaves (in MacDonald, 1963).

In woman, Rydberg also noticed that the crystals are composed of sodium chloride, but their forms are determined by proteinaceous matrix (in Kihlström & Fjellström, 1969). The crystallizing cervical mucus is composed of 97% ash of sodium chloride (NaCl). The presence of estrogens, mucoproteids, sodium (Na) and potassium (K) ions is very needed for the crystallization. The protein molecules, which are included in a crystalline nucleus, bind the molecules of water together with salts Na and K. The crystals are created by these salts in the fibers of protein molecules, this way the crystalline patterns are formed (Rob & Stehlík, 1983).

3.2.1. Crystallization of cervical mucus

The structure of the cervix in the cows, ewes and goats is much simpler and manner than the cervix in women (Heydon & Adams, 1979). The cervical mucus plays a very important role in cervical function. It controls and directs sperm migration (Mattner, 1966; Davajan et al., 1970).

The cervix plays an important role as a barrier or filter in fertilization and reproduction (Vaissaire, 1977; in Silva et al., 1995). The accurate estrus detection is an important factor for reproductive management. The crystallization of cervical mucus was researched in many species by different authors. Also many authors described the cyclic changes of cervical mucus as related to the menstrual cycle (Wollner, 1938). The cervix and its secretion have important influence on the reproductive success of mammals (Mattner, 1973; Hafez & Kanagawa, 1972 in Zaaijer, 1993). The statement of crystallization dependency on blood estrogen and progesterone concentration was described in Heydon & Adams (1979). Papanicolaou (1945) described the argyrophil secretion in cervical mucus and its distinct rate near the peak of follicular activity at the time of ovulation (in Salvatore, 1961). Alliston et al. (1958) demonstrated the cognition of Rydberg (1948) about relationship between chlorides and crystallization. Also Moghissi (2002) mentioned dependability arborization of cervical mucus on concentration of electrolytes, especially sodium chloride (NaCl). In the study of Linford (1974) were described the maximum values of crystallization patterns during estrogen dominance and minimum of these values during progesterone dominance.

Bone (1954) and Zondek (1954) mentioned that the contamination (e.g. electrolytes, cellular debris) of cervical mucus may lead to incorrect observations by influencing the crystallization (in Raeside & McDonald, 1959). Zondek (1953) pointed out

that if the cervical mucus is blended with blood (in cases of erosion of the cervix or intrauterine bleeding), it cannot be utilized for observation of crystallization, because blood has inhibiting influence on creating crystals (in Zondek & Rozin, 1954).

The cervical mucus was tested in animals and also in human. The phenomenon crystallization was observed in following mammals; cows (Ahmadi et al., 2005; Gündoğan, 2009; Ježková et al., 2008; Alliston et al., 1958), pigs (Betteridge & Raeside, 1962; Haynes, 1971), ewe (Raeside & McDonald, 1959), mares (Dinger & Noiles, 1982) and rhesus monkey (David & Mastroianni, 1968). In all of the researches the samples of cervical mucus were smeared on the glass slides and left to dry at room temperature. Lamond and Shanahan (1969) left to dry samples under an infrared lamp at 40°C. The sampling methodology differed according to authors. In cows cervical mucus should be collected by an aluminium speculum with plastic tube (Noonan et al., 1975), a plastic inseminating tube (Alliston et al., 1958), a pipette (Ježková at al., 2008; Lamond & Shanahan, 1969), an inseminating catheter and glass vaginal speculum (Ghannam & Sorensen, 1967). In rhesus monkey the cervical mucus was obtained by a cotton-tipped applicator (David & Mastroianni, 1968). In ewes sampling was done by glass rod or aspiration (Raeside & McDonald, 1959).

The changes in bovine cervical mucus was noticed by Noonan et al. (1975), when the samples of cervical mucus was obtained from cows during the estrus cycle and early pregnancy. The crystalline changes were monitored at the time of estrus.

Alliston et al. (1958) studied the crystallization of cervical mucus in heifers. Each of samples was evaluated by different author and the final classification was defined by the median of the values assigned or by agreement of at least two authors as to its classification. The crystalline patterns were calculated according to the following score: (1) No patterns. (2) Patterns around bubbles only. (3) Typical patterns, but covering less than one half of the total slide area. (4) Typical patterns, covering more than one half of the total slide area, but not complete. (5) Typical patterns over complete slide area, both short and long ferns present. (6) Typical patterns over complete slide area, only long ferns present.

In the study of Gündoğan (2009) the crystallization of cervical mucus was positively connected with vaginal hyperemia, vulva edema and ovulatory follicle size.

Ahmadi et al. (2005) researched the crystallization of cervical mucus in correlation with parturition. The samples were obtained from cows in late gestation. The crystalline

patterns were scored (0-4) according to Tsiligianni et al. (2001): (0) no crystals, (1) atypical crystals, (2) many atypical and few typical crystals, (3) many typical crystals and few atypical crystals, (4) only typical crystals.

The influence of crystallization of cervical mucus was tested in cows by Ježková et al. (2008). The samples of cervical mucus were obtained on the day of insemination.

The crystallization of cervical mucus as a means of early pregnancy diagnosis in cows was demonstrated by Ghannam and Sorensen (1967). The crystallization was evaluated according to shape and extent of crystallization. Four groups of crystallization were determined: very-marked patterns were characterized by heavy crystallization, the crystals were long ferns; marked patterns were characterized by short, curved, feather-like structures; mixed patterns were represented by the presence of some fern-like crystals accompanied by areas lacking crystallization; negative group was typical by absence of crystallization. The variations in crystallization were noticed according to the phase of estrus cycle in which the samples were collected.

Lamond and Shanahan (1969) studied the changes in cervical mucus from normal and ovariectomized cows treated with hormones. The crystallization was scored in the following ways: (0) no presence of crystallization or incomplete and complete crystals over less than half of the area; (1) crystalline patterns over greater part of slide, subvenation indistinct, short and incomplete crystals; (2) elongated crystals, clearly defined venation and subvenation over the whole area. The ratios of chloride to total protein (CMIt) and chloride to ″residual″ protein (CMIr) were calculated.

The cervical mucus as an indicator of ovarian activity was also tested in pigs by (Betteridge & Raeside, 1962). The crystallization was evaluated according to the following score: negative – no crystallization detectable; weakly positive – very occasional and scattered patterns; positive – occasional but well formed patterns or patterns seen in most fields; strongly positive – well formed patterns seen in most fields.

The research of cervical mucus in rhesus monkey was made by David and Mastroianni (1968). This study was focused on the correlation between ovulation and status of the cervical mucus.

Raeside and McDonald (1959) studied ewes for the crystallization of cervical mucus. The cervical mucus was obtained from cycling and pregnant animals.

The results of different studies focused on cervical mucus in cows showed the relationship between crystallization and estrus cycle or pregnancy. Alliston et al. (1958)

published that the rate of crystallization increased gradually to a maximum at the time of estrus and decreased to the average time of ovulation. According to Ježková et al. (2008) the best results in pregnancy rates were noticed in cows where the ferny-like crystallization was detected. The highest motility of sperm was observed in the case of club moss-ferny like and ferny-like crystallization. Ghannam and Sorensen (1966) discovered that the very-marked crystalline patterns were monitored as early as three days before estrus and consequently these crystals continued as long as seven days after estrus. In correlation with estrus, these patterns appeared from five days before estrus to eight days after estrus. The normal crystalline patterns occurred in animals with silent heat. The crystallization in the pregnant cows varied between mixed and negative type. Some of marked patterns were watched. The pregnancy of cows was determined on the basis of the absence of a very marked crystallization and the presence of a positive vaginal pressure after injecting oxytocin. In their results was mentioned, that the cow was non-pregnant and still cycling during presence of very-marked crystallization. On the other hand, marked, mixed or negative crystallization showed that the cow was either pregnant or non-pregnant. In Lamond and Shanahan (1969) the minimum values of crystallization and chloride concentration were observed on days 4-19 of the cycle. Contrary, the maximum values of crystallization and chloride concentrations were from one day before estrus to 2-3 days after estrus. The cervical mucus index was evaluated as a better indicator of estrus than crystallization or chloride concentration. As for parturition, Ahmadi et al. (2005) published that the significant growth of crystals was present from day 8 to term. In compliance with Noonan et al. (1975) there was a suggestion, that cervical mucus was better indicator of reproductive status than vaginal mucus. According to Alliston et al. (1958), there was a possibility to use the crystallization of cervical mucus as a means of estrus detection.

The results of arborization in other animals showed, that in pigs the peak of crystallization was observed two days before onset of estrus. The presence of crystalline patterns in pregnant animals was very low. At early pregnancy, no crystallization was watched (Betteridge & Raeside, 1962). The peak of crystallization in rhesus monkey was observed one day before ovulation. On the other hand, the extensive decline of crystallization was noticed in the subsequent day (David & Mastroianni, 1968). According to the results of Raeside and McDonald (1959), mixing cervical mucus with blood, vaginal mucus, lubricant cream and seminal plasma had the negative influence on the crystallization. The crystalline patterns were recorded near estrus in all abnormal cycles.

During the silent heat, anoestrus and pregnancy the crystallization was also observed. In normal cycling animals, the crystallization was detected from three days before estrus until two days after estrus. In all ewes the crystallization was occurred on the day before and on the day of estrus.

The mechanism of crystallization in human was reported by Zondek in 1953 (in Zondek & Rozin, 1954). In protein solutions which contain electrolytes the presence of crystallization was confirmed. Substances, such as serum albumin, ovoalbumin, fibrinogen, beta and gamma globulin, peptone, dipeptide, tripeptide and polypeptide, were discovered to create this phenomenon upon admixture with certain electrolytes. Such electrolytes include sodium chloride, potassium bromide. However other salts (such as calcium chloride, barium chloride, sodium bromide, potassium nitrate, sodium sulfate and sodium iodate) failed to bring the crystalline reaction (Zondek & Rozin, 1954).

Zondek (1959) presented that in monosaccharides and polysaccharide mixed with saline or dissolved in this solution the crystallization was attended. Also protein or carbohydrates in cervical mucus created crystals after the contact with the proper electrolytes.

The various methods could be used for collecting samples of female cervical mucus: aspiration (Zondek & Rozin, 1954) or a cotton-tipped applicator (Forman, 1956). The obtained samples were smeared on a glass slide and left to dry at room temperature (Zondek & Rozin, 1954) or passed through a flame (Zondek & Rozin, 1954) or over a Bunsen flame (Forman, 1956).

The crystallization of cervical mucus was tested in correlation with different factors. Garcea et al. (1984) researched the crystallization of cervical mucus in correlation with mucus canalization. Harvey et al. (1960) tested the relationship between consistence of cervical mucus and its crystallization. In many researches the value of cervical mucus was studied in connection with ovulation timing and the sensitivity of mucus to the sperm, in the diagnosis of pregnancy, and in the estimation of placental insufficiency (Wearing, 1959). In Zondek and Cooper (1954) castrated women were tested, in cases of amenorrhea and during the postclimacteric period, the crystallization was observed 3-4 days with estrone 1mg, estradiol 0.3mg, or estradiol benzoate.

In Zondek and Rozin (1954) the crystallization was classified according the score: negative – no typical crystallization, 1 plus – presence of typical crystallization in only a few places, 2 – typical crystallization present in several places in a field, 3 plus – distinctly

positive crystallization, with most of the dried mucus in any field in a state of typical crystallization. The implementation of the crystallization test depends on the different conditions. The function of corpus luteum and, thus, ovulation, were verified by the strongly positive (3 plus) crystallization test. Urdan and Kurzon (1955) divided arborization to three categories, namely light, moderate and heavy.

In research of Harvey et al. (1960) the consistency of cervical mucus was scored from 0 to 21. No crystallization was present in mucus with consistency higher than 7.

The crystallization was evaluated by Wearing (1959) only as positive (total and typical crystalline patterns in the whole field and over the whole slide expect) and negative (no total typical crystallization). The mucus crystallization was tested during menstrual cycle – ovulatory cycle, anovulatory cycle, during pregnancy and at the menopause. Campoz da Paz demonstrated that in the anovulatory cycle the typical crystals were present in the second half of the cycle (in Wearing, 1959).

During pregnancy the cervical mucus was studied by Zondek and Cooper (1954). After application of injection of 10mg of estrogens the cervix was open and the positive crystallization was detected similar to that monitored during the time of ovulation. After mixing dried mucus with highly diluted salt solutions (for example NaCl, KCl) the crystallization of this mucus during pregnancy was showed. In the study of these authors the crystalline patterns in early pregnancy were considered.

As for conclusions, Zondek and Rozin (1954) published that the utilization of crystallization test was reject in the lightly presence (1 plus) of crystallization before menstruation. The impossible utilization of test for determination of the corpus luteum function was confirmed by negative presence of the crystallization in the intermenstruum. The results showed, that the presence of salts is essential, dialyzed cervical mucus failed to create crystals, but the crystallization was observed after mixing mucus with electrolytes. Also the mucus obtained during postmentruum, prementruum, postmenopausal stage and pregnancy did not show the crystalline patterns. The test of crystallization was considered to be utilized as a diagnostic mean for assessing the function of corpus luteum. The result of Garcea´s et al. (1984) study showed, that the canalization had increasing potential daily, as same as estradiol, but the potential of crystallization was on the same value for a long time. In Harvey et al. (1960) the peak of crystallization was observed 16 to 17 days before next period. In the study of Forman (1956) was tested the crystallization of cervical mucus as a quick and easy examination of estrogen activity. The test of crystallization studied by

Forman had negative potential during the postmenstrual phase due to insufficient estrogen influence and inhibiting activity of progesterone. Near start of ovulation the crystallization effect became distinctive. The phenomenon of crystallization examined during midcycle and preovulatory phase was considered as a quite exact test for ovulation. As a mean of assessing the time of ovulation was also assumed the daily monitoring of cervical mucus. Wearing (1959) noticed that after the menstrual period no crystallization was observed. Much less significant crystals were visible near the end of cycle. This study also verified the presence of typical crystallization during mid-cycle. Disproving of the pregnancy was indicated by typical crystallization of cervical mucus. The negative evidence of typical crystals in samples of cervical mucus was observed during pregnancy. The reduced occurrence of crystallization during pregnancy was influenced by a persistence of corpus luteum. The cervical mucus smear may be used as a test for predicting the pregnancy. The menopause period was presented by negative occurrence of crystallization. Zondek and Cooper (1954) assumed the test of crystallization as a possibility of excluding pregnancy. The diagnosis of pregnancy was confirmed by strongly positive crystallization of cervical mucus contrary to the diagnosis of tumor.

3.2.2. Saliva crystallization

The saliva crystallization was mainly examined in women (Kullander & Sonesson, 1965; Barbato et al., 1993; Guida et al., 1993; Berardono et al., 1993; Pattansuttinont et al., 2007).

The saliva sampling should be done by catheter (Kullander & Sonesson, 1965) imprinting a frosted glass slide over the tongue or with a finger in glove (Pardo-Carmona et al., 2010) and by clean coffee stirrer (Haberová, 2010). The samples were smeared on the glass slide and left to air dry at room temperature (Kullander & Sonesson, 1965; Barbato et al., 1993; Pardo-Carmona et al., 2010; Haberová, 2010).

The saliva crystallization in female was examined in different stage of reproductive cycle. Kullander and Sonesson (1965) obtained saliva from normally menstruating, pregnant and post-menopausal women.

In Guida et al. (1993) was hypothesized that estrogens, catecolestrogens and opioid tone have a positive influence on the saliva crystallization. In comparison with Berardono et al. (1993) who described the leaf crystallization of human saliva as a non-specific phenomenon, independent on the action of estrogenic hormones.

Pattansuttinont et al. (2007) tested infertile women. For five days were given 100mg of comiphene citrate and the saliva were collected daily until seven days after ovulation. The saliva samples were graded from 1-3, according to their extent and intensity.

The studies focused on changes in saliva crystallization in animals are very limited. The saliva crystallization in bitches was observed by Pardo-Carmona et al. (2010) and the presence of saliva in camels (*Camelus bactrianus*) by Haberová (2010).

In the study of Pardo-Carmona et al. (2010) were used adult bitches. The saliva were scored according the classification: type 0 no evidence of crystallization, type 1 when the presence of partial crystallization was noticed, i. e. when no total crystallization was evident but an initial crystallization was visible on a part or whole surface of the glass slide or when the samples showed patterns with long stems and clear venation on a part or whole surface of the slide, with stem-like fir leaves and type 2 when total and clear crystallization was observed with stem-like bold fern leaves.

Haberová (2010) studied the saliva of Bactrian camels (*Camelus Bactrianus*). In comparison with Pardo-Carmona et al. (2010), where the crystalline patterns were described according to their coverage and length of stems, in Haberová (2010) the crystallization was classified according to its appearance. The score system was described as: 0 (none or dotted), 1 (branch-like), 2 (mixed branch-like and fir-like), 3 (fir-like), 4 (mixed fir-like and branch like), 5 (fern-like), 6 (reticulated) and 0.5 (mixed fern-like, reticulated, dotted and branch-like).

As for conclusions of all mentioned studies, the crystallization patterns of the saliva from pregnant women showed coarser structure than the saliva from menstruating women. The coarser crystallization was also noticed in women after menopause and in one case no crystallization occurred at all (Kullander & Sonesson, 1965). Barbato et al. (1993) verified the dependability of the salivary crystallization method of ovulation detection. Nonfertile period was characterized by no crystallization. Limited crystallization showed the beginning of the fertile period. According to the results, a direct correlation between salivary crystallization and the phase of reproductive cycle was verified. Also Guida et al. (1993) described the saliva crystallization in connection with the menstrual cycle in women. In Pattansuttimon et al. (2007) were determined two peaks of median saliva crystallization scores; one was two days prior ovulation and the other was five days post ovulation. No correlation between the peak of saliva crystallization and day of ovulation

detected by ultrasound was observed. The results showed that in clomiphene citrate-stimulated cycles was not found coherence the crystallization of saliva with ovulation. Berardono et al. (1993) noticed the crystalline structures in saliva of male.

The observation of the saliva crystallization was considered a new technique for monitoring reproductive cycle (Guida et al., 1993). But according to the results of Berardono et al. (1993) the saliva crystallization was not recommended as a reliable method to establish the woman fertile period.

In case of animals, the results in bitches showed that saliva crystallization (type 1 and 2) appeared some days before and after the optimal time for mating. The saliva arborization lasted for several days, appearing before and after the expected day of ovulation. The variations in saliva crystallization were noticed during the follicular phase. This study was considered as a supplement of other methods of determining the fertile period in bitches, but its potential capacity was demonstrated. In Bactrian camels (*Camelus Bactrianus*) all types of crystallization, which were mentioned earlier, were noticed in saliva as well changes in crystalline patterns.

3.2.3. Crystallization of other body fluids

The crystallization is not specific only for cervical mucus and saliva. It can also be created in the other mucus secretions and body fluids. The crystallization was reported in vaginal fluid (Dinger & Noiles, 1982; England & Allen, 1989; Noonan et al., 1975), nasal mucus (Peterson, 1984; Zondek, 1959), aqueous humour (Liotet et al. 1987; in Golding & Brennan, 1989), cerebrospinal fluid, fluid from follicles, hydrosalpinx and in ovarian cysts (Zondek, 1954; in Golding & Brennan, 1989). Golding and Brennan (1989) described the basis of crystallization in tears. The crystals were also observed in colostrum, milk and semen (Zondek & Rozin, 1954). The Cowper´s fluid from human bulbourethral gland was researched by Berthou and Chretien in 1995.

In presented studies the samples of different body fluids were smeared on the glass slides, left to dry and evaluation was done microscopically (Tabbara & Okumoto, 1982; Norn, 1987; Dinger & Noiles; 1982; Noonan et al., 1975; England & Allen (1989). In one case the monitoring of crystallization was made under the phase-contrast microscope (Golding and Brennan, 1989).

In 1982, Tabbara and Okumoto reported the qualitative test for tear mucus deficiency based on the presence or absence of crystals in conjunctival scrapings from interior ocular conjunctiva.

The crystallization in nasal mucus was described by Peterson (1984). The crystalline patterns of nasal mucus in women, with regular menstrual cycle, were studied here. The changes in nasal mucus were tested in correlation with changes in basal body temperature and phase of menstrual cycle. The degree of crystallization was scored from 1 to 4.

Norn (1987) observed two quantitative pipette samples and smears from eyes. Two types of crystallization were detected. His study was designed on the crystallization in conjunctival-cytologic preparation**.**

The variations in tears mucus crystallization was recognized and classified for the first time by Rolando in 1984. Rolando´s classification system for tear mucus crystallization is based on the size and spacing between crystals (in Pensyl et al. 1998). The study of Pensyl et al. (1998) was focused on the examination the intraobserver and interobserver repeatability. According to the results, the Rolando´s scoring system was considered as a means of tear crystallization classification.

The correlation between the crystallization in vaginal mucus and the teasing in mares was examined by Dinger and Noiles (1982). The vaginal mucus was tested as a method for detecting estrus. The samples were obtained by the suction and swab methods. The mucus taken by the suction method technique created crystallization more effectively than mucus collected by the cotton swab technique.

Noonan et al. (1975) described changes in vaginal fluid together with cervical mucus during estrus and early pregnancy in cows.

The changes in crystallization of anterior vaginal fluid in bitches were monitored by England and Allen (1989). The animals exhibited a typical crystallization during the pro-estrus and estrus. The vaginal fluid was collected daily from anoestrus animals. In this study, the fluid was obtained using an adaptation of the method of Allen and Dagnal from 1982; a sterile insemination pipette was applied. A syringe was used for the suction. The crystallization was previously identified and divided into five classes from minor crystallization (short stems and irregular scattered stellate) to bold crystallization (long stems and clear venation and subvenation). Crystalline patterns were compared with the photographic records and scored in interval 0-5. According to the surface area covered by

crystals the samples were classified by following criteria; up to 1% cover (score 1), 1-5% (score 2), 5-10% cover (score 3), 10-20% cover (score 4) and over 20% cover (score 5). This information of crystallization score and cover score were added to give the ″Ferning Index″. The changes in the Ferning Index during the estrus period were noticed (England & Allen, 1989).

The crystalline patterns were also observed in the seminal fluid (Kihlstöm & Fejllström, 1969). The study was focused on a similar cyclic variation in the pal-leaf-like crystals of the seminal fluid. Also was tested the correlation between the crystalline patterns and the concentrations of proteins and some inorganic ions in the seminal fluid. The semen from rabbits and bulls was obtained by artificial vagina and from human by masturbation. The samples were freed from spermatozoa in a centrifuge.

According to the results of Peterson (1984) no relationship between the nasal mucus crystallization and the basal body temperature was noticed. It was significant that as the temperature increased, the stage of crystallization also increased. Also no correlation between the nasal mucus crystallization and the menstrual cycle was observed. Norn (1987) confirmed the presence of crystalline patterns in 29% of pipette samples from eyes. This effect was most often in samples with fairly large amounts of mucus, more frequent in neutrophilia than in lymphocytosis, and the rarest in normal cytology. In the study of Dinger and Noiles (1982) the vaginal mucus crystallization was not evaluated as the effective method for the detecting estrus in mares as using a teaser stallion. Noonan et al. (1975) reported that the dry matter concentration of vaginal mucus reached a minimum value on the day of estrus and a maximum value at midcycle. From the day of estrus to the 19^{th} day of pregnancy the dry matter concentration of vaginal mucus increased and the crystallization decreased. England and Allen (1989) concluded when crystallization in vaginal fluid was not created the bitches were in late estrus. This study showed the greatest crystallization between 1 and 5 days after the maximal estrogen concentrations. This was considered as a correlation with the period before or at ovulation when follicles were becoming luteinized. The vaginal mucus crystallization was regarded by Abusinea (1962) and Alliston et al. (1958) to be more irregular during the estrus cycle than crystallization of cervical mucus (in Noonan et al., 1975). As for semen, the crystallization was detected in about 50% of the ejaculates from bulls and men and in the larger majority of samples of rabbits. No correlation between the concentrations of potassium and the type of crystals was observed (Kihlstöm & Fejllström, 1969).

4. Materials and Methods

4.1. Bibliographic research

The keywords connected with this topic were used to obtain available resources, focused on the crystallization. The researchers use different terms for the crystallization. It was needed to complete these terms. For this study were used following key words – crystallization, crystalline patterns, ferning, arborization, crystalline structures, cervical mucus, vaginal mucus, nasal mucus, saliva, body fluids, reproductive cycle, heat detection, fertile period, ovulation, pregnancy. The searching was done by combination of these key words.

The citations were done according to Guidelines of journal "Biology of Reproduction" (Society for the Study of Reproduction, 2008).

4.2. Animals

Eight Holstein dairy cows from the Czech University of Life Sciences Prague (CULS) Farm Estate Lány-Ruda were chosen for present study. There were kept under similar conditions of nutrition and management. The age of animals was two to three years with the first calving in 2011. All cows were found to be free of any abnormalities in their genital system and reproductive cycle.

These animals were included into hormonal estrus synchronization program. The process of synchronization started on August 9th 2011, when three milliliters of Supergestran (NORDIC Phrama, s.r.o.) were applied. The application was repeated on August 16th. Three milliliters of Remophan (Bioveta, a.s.) were used one week later. On August 26th tested cows were inseminated.

4.3. Sample collection

This experiment was based on the method presented by Haberová (2010) as was mentioned in chapter 3.2.2. (Saliva crystallization).

Samples of saliva from eight Holstein cows were obtained daily during the time period from 10th August to 29th September 2011. So the saliva crystallization was monitored during 16 days before insemination and 34 days after insemination. Sampling

was done after the first morning milking. Tested cows were milked preferentially. The animals were taken away into the separated boxes after milking, where they were subsequently fixed. Cows did not have access to feed. Saliva production was stimulated by the hand inside the mouth. The samples were collected from the part under upper and lower lip. Care was taken to avoid causing any stress to the animals. Each sample was taken by new and clean coffee stirrer (Fig. 1). Also the glass slides were washed and degreased before. Consequently the saliva were smeared in thicker layer only on a center of the slide and allowed to air dry at room temperature for a few minutes. After that the smears were put into the box and once a week transported to the laboratory where were tested for the crystallization.

Fig. 1: The coffee stirrer for saliva sampling (photo by author)

4.4. Evaluation of samples

The samples were assessed once a week. The evaluation of crystallization was done by a light microscope at magnification 400x. Fig. 2 illustrates the system of searching crystals in slide.

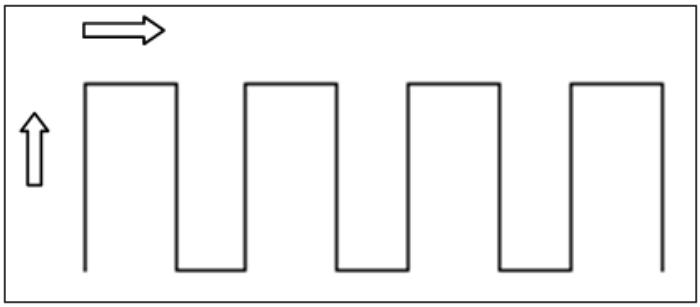

Fig. 2: System of searching crystals on the glass slide.

Crystals were classified according to the adapted system by Haberová (2010). For the first time the presence and type of crystallization was observed and noted. If the presence of crystallization was confirmed, its type was also described by a scoring system made by Haberová (2010). The list of types of crystallization with abbreviations is shown in table 1.

Tab. 1: General types of crystallization

Type of crystallization	**Abbreviation**
None	0
Branch–like	BL
Mixed branch–like and fir-like	BL+FIL
Mixed branch-like and fern-like	BL+FEL
Fir-like	FIL
Mixed fir-like and fern-like	FIL+FEL
Fern-like	FEL
Atypical	A
Dotted	D
Mixed branch-like and fir-like and fern-like	BL + FIL + FEL

Subsequently the characteristics of different crystals were also examined. Among these features include thickness and entirety of crystals, which are further divided into three categories of thickness: score 1 = thin, score 2 = medium thick, score 3 = thick. For entirety was used following score: 1 = entire, 2 = separated, 3 = broken crystals.

The density of crystals was also classified. It was evaluated according to the scale 10-20-30-40-50, which means that the smallest density is characterized by the number 10 and the biggest density by the number 50.

Finally sample quality was examined, because it was necessary to assess and take to cognizance the possibility of contamination of the samples. The quality of samples was evaluated subjectively and following terms were used for description of samples quality: good quality (OK), contaminated sample (C) and thin smear of saliva. All characteristics and values were noted to table (see in appendix 1).

Only one general type of crystallization was described in one sample.

4.5. Classification of crystallization

The classification of presented crystalline patterns was based on searching of similar features between saliva crystallization in camels and cattle.

The branch-like crystals (Fig. 3) were assessed as irregular patterns, where the main branches were significantly longer than side branches. The connection between individual branches was asymmetric and also angle of fusion was different.

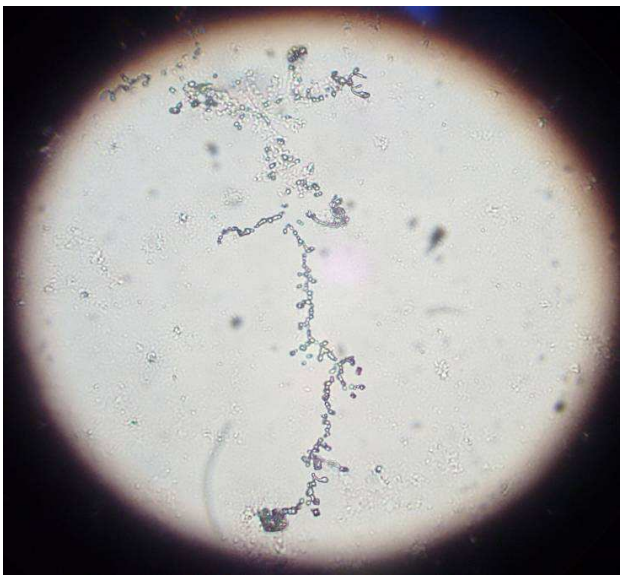

Fig. 3: BL crystallization (photo by author)

The determination of fir-like crystallization was more complicated than in case of branch-like crystallization. According to example from score system (made by Haberová, 2010) the fir-like crystals (Fig. 4) remind branch of fir, thus the connection of side branches had as same structure as needle of fir. Contrary fir-like crystallization in cattle was decided on the basis of search for similar features between example from score and patterns in cattle saliva. The deciding factor in case of fir like crystallization was acute angle of connection side branches to main branch, because fir-like crystallization is represented by noticeable main branch. Thus the crystals were marked as fir-like if the symmetric side branches were connected to main branch under acute angle.

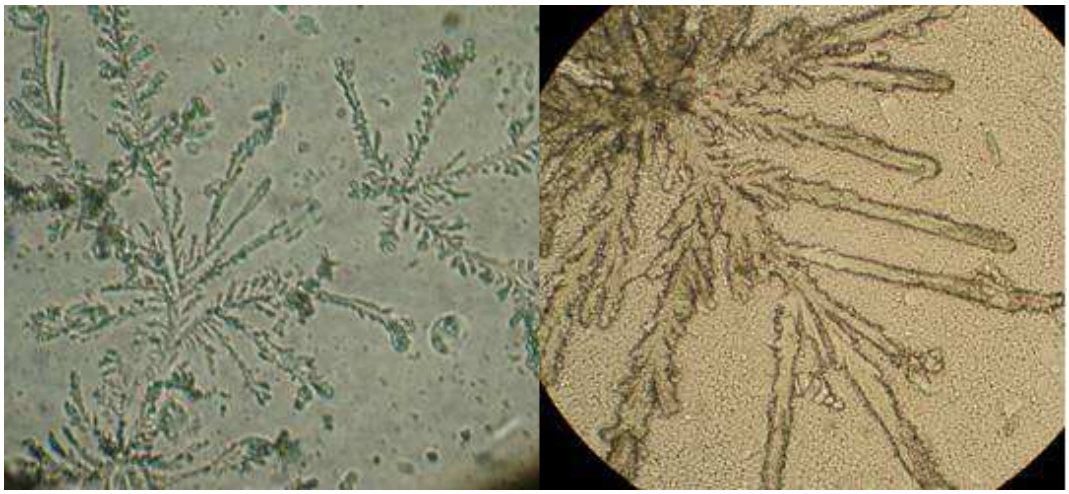

Fig. 4: FIL crystallization (photo by author)

The determination of fern-like crystallization was also based on link between side and main branches. The perpendicular connection between branches was typical for fern-like crystals, which are displayed in Fig. 5. But fern-like patterns in cattle saliva did not remind typical fern leaf, because the connection of individual side branches was very sparse and the branches had irregular length in comparing with fern leave.

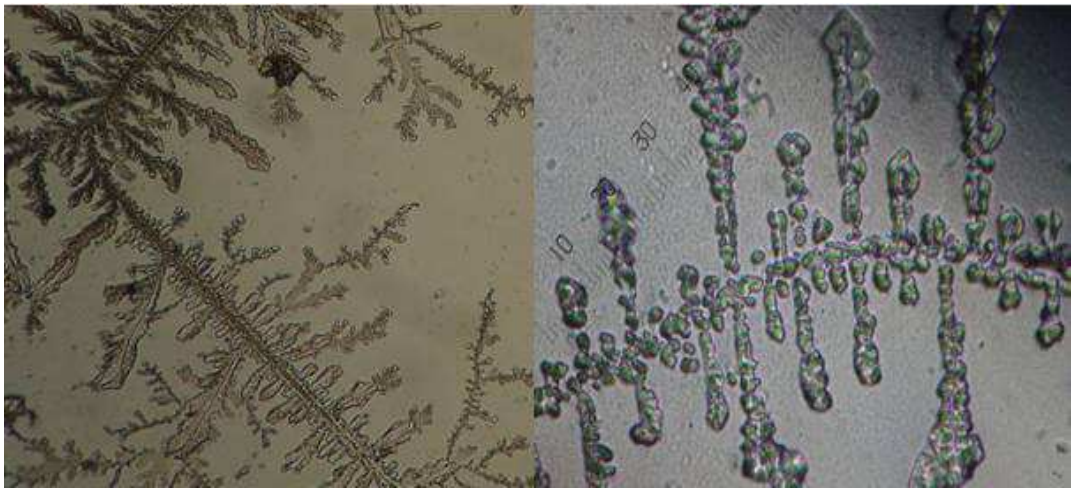

Fig. 5: FEL crystallization (photo by author)

All of mentioned individual types of crystallization created the combinations. The mixed branch-like and fir-like and fern like was defined when all of three types were noticed. The fern-like patterns were represented with the lowest frequency and any separated fern-like crystals were not detected.

If the branch-like crystallization was supplemented with fir-like features the crystals were characterized as mixed branch-like and fir-like (Fig. 6). This type of crystallization was also represented by equal ration between branch-like and fir-like crystals.

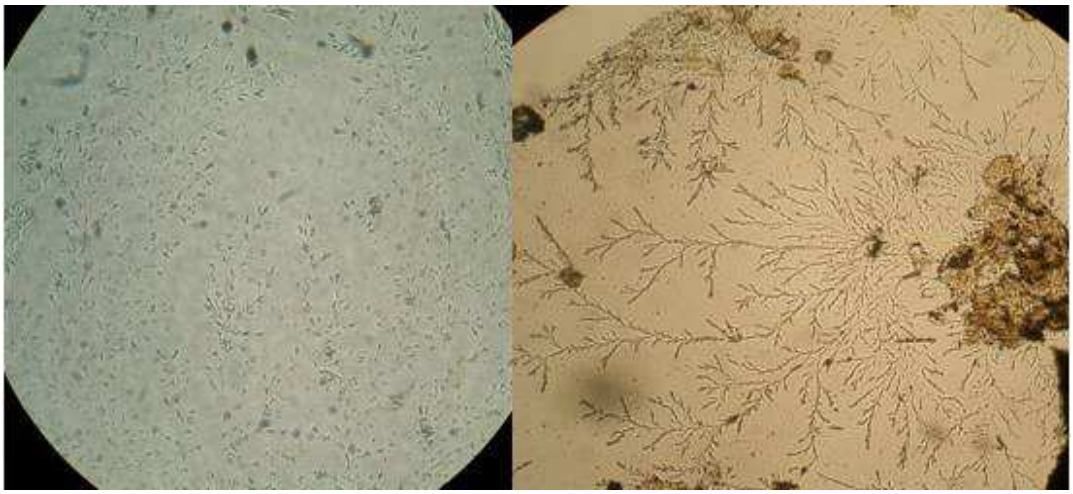

Fig. 6: BL + FIL crystals (photo by author)

The branch-like crystallization was also detected together with fern-like patterns. This combination was noticeable only by presence of fern-like signs. Thus when the mixed branch-like and fern-like crystallization (Fig. 7) was determined there were not noticed any separated fern-like crystals.

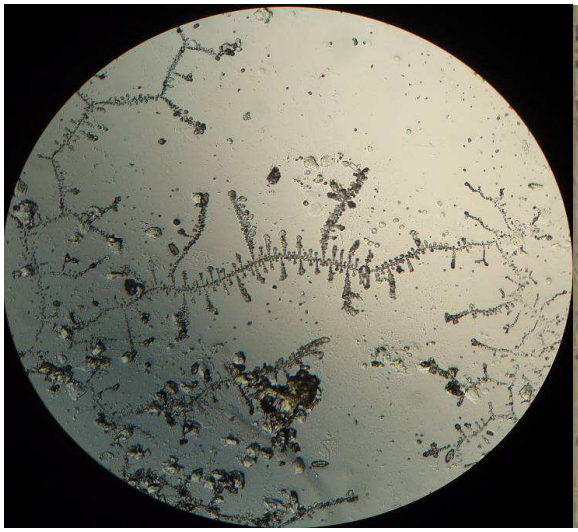

Fig. 7: BL + FEL crystallization

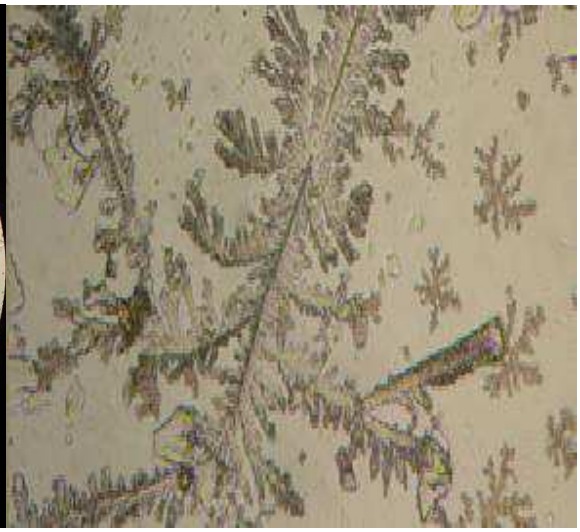

Fig. 8: FIL + FEL crystals (photos by author)

The mixed fir-like and fern-like crystallization (Fig. 8) created the most developed crystals almost every with basis from one point. The determination of this type was based on presence of fir-like and fern-like patterns which were connected to one crystal. Sometimes fir-like crystals were separated, but fern-like features were only as supplements of crystallization.

4.6. Sample staining

The Giemsa stain was used for coloring of samples. The process of sample coloring was performed according to recommendation of Rajmon (2011). Before usage the stain had to be warm up. It was done by dilution with hot water in a ratio 1: 9, in a beaker of 100ml, where the majority was represented by water. The solution was poured into vessel of coloring equipment, which is displayed in Fig. 9 For coloring were selected five samples with large and well evident crystals. Before staining were noticed coordinates of crystals on the glass slide. Consequently the glass slides were put to holder and dipped into vessel with Giemsa solution for 30 seconds. Immediately after coloring the glass slides were

quickly rinsed under running water and left to dry at room temperature (Fig. 9). In case of stained glass slides were evaluated only noticed parts of sample.

Fig. 9: The equipment for staining and drying of samples (photo by author)

4.7. Statistical analysis

The statistical analysis was realized in program STATISTICA CZ 10.0 (StatSoft, Inc. 1984-2011). The general types of crystallization (Tab. 1) were used for majority statistical tests. Other generalization of maximal type of crystallization was used for easier assessment. Thus in statistical evaluation of combination of types of crystallization were examined higher levels of crystallization, where was determined following system: FEL>FIL>BL. For example, when mixed branch-like and fir-like crystallization was detected the fir-like crystallization was integrated into statistical analysis. The atypical crystallization was also included into statistical evaluation. When was atypical arborization detected separately it was statistically assessed as atypical crystallization. Contrary when atypical crystals were observed together with general types of crystallization these types were included to statistical analysis and atypical crystallization was noticed only in notes.

The occurrence of type of crystallization (before and after insemination) was evaluated by contingency tables and the data distribution was tested by Pearson´s chi-square test. The Kruskal-Wallis ANOVA and multiple comparison test were used for assessment of additional characteristics.

The differences in crystallization in pregnant and non-pregnant animals were evaluated by Kruskal-Wallis ANOVA. The control of results from this test was realized in contingency tables. The changes in arborization after insemination were displayed and evaluated after each five days.

The individual stages during synchronized cycle with the most common crystalline patterns were described separately. Also the pregnancy detection was evaluated separately.

5. Results

From available resources was found out, that the saliva crystallization in cattle have never been elaborated.

For evaluation of saliva crystallization in cattle 408 samples were observed in total. The arborization was confirmed in all tested animals.

The determination of pregnant and non-pregnant animals was notified by livestock specialist from CULS Farm Estate Lány – Ruda. The pregnancy was confirmed in five animals. The occurrence and characteristics of arborization were separately described during synchronization of estrus and in pregnant and non-pregnant animals.

The changes of saliva crystallization were noticeable during period before insemination and also in pregnant and non-pregnant animals. These modifications were based on size of crystals, crystalline patterns, type of ferning, level of definite crystallization, interconnection between individual crystals and occurrence of crystallization. The majority of crystals in cows based from one point and created irregular "stars".

5.1. The evaluation of occurrences of individual types of crystallization

The results from statistical evaluation showed difference in distribution of data in various stages of experiment (Pearson´s chi-square test: 145,863, DOF = 36, $p < 0.00001$). The occurrence of basic individual crystalline features (maximal types) is displayed in Fig. 10, but these features were detected also in combinations.

The branch-like crystals had different form in structure. Some of them were resembled to long and branched "chain". On the other hand branch-like crystals looked like indefinite formation. The variations in size and development of branch-like crystals could be considered as the changes of crystallization during whole synchronized estrus.

The branch-like crystallization was among three most common types (36.27%). The occurrence of branch-like crystals was quite similar in both stages after application of preparation of synchronization (22.30% after 1st application vs. 22.97% after 2nd application). The size of branch-like patterns was different in period before insemination. Many days before insemination the crystals were smaller than crystals immediately before

insemination. The occurrence of branch-like crystallization was more noticeable in pregnant animals (38.51%) than in non-pregnant animals (16.22%).

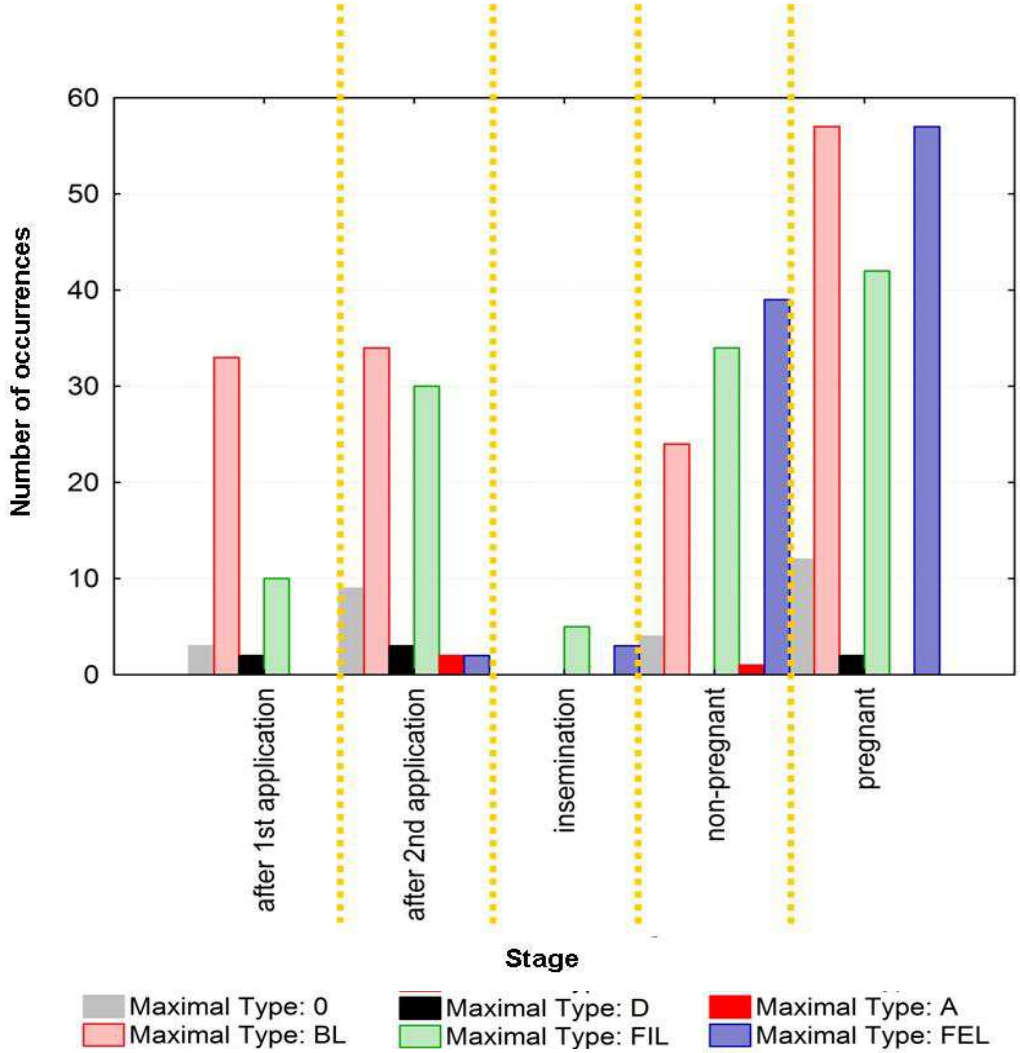

Fig. 10: The graph of occurrence of individual crystalline patterns

The fir-like crystallization was monitored mainly in combination with other types but itself was recorded after the first application of preparation of synchronization. Higher occurrence of fir-like crystallization was recorded after the second application than after the first application (9.76% vs. 2.44%). The total frequency of this type was more often marked in pregnant animals than in non-pregnant animals (53.66% vs. 34.15%).

The fern-like crystallization was mostly occurred in combination with other type. The first presence of fern patterns was noticed after the second application of preparation of synchronization but only in combination with other crystallization. The occurrence of

fern-like arborization without other type of crystals was marked in period after insemination with higher occurrence in non-pregnant animals (66.67% vs. 33.33%).

Also the mixed arborization differed in size, features of fir-like crystallization and connection of individual branches. The branch-like patterns were represented by higher density of individual branches.

The mixed branch-like and fir-like crystallization was also among three most common crystallizations (19.61%). Its dominance was better marked after the second application of preparation of synchronization than after the first application (32.50% vs. 11.25%). From pregnancy point of view, the occurrence of this type of crystallization had same level in pregnant and non-pregnant animals (25% vs. 25%).

The mixed branch-like and fern-like crystallization was noticed after insemination only in non-pregnant animals and with very low frequency (0.25%).

Other type of combined crystallization was described as mixed fir-like and fern-like. It could be considered as the highest level of combination between two types of crystallization. The mixed crystallization was observed after insemination with higher frequency in pregnant animals (61.90% vs. 38.10%). This arborization was among three most occurred types (20.59%).

Some of crystals or combined crystals were supplemented with the atypical crystallization. The atypical arborization was detected in 0.74% separately. Also it was discovered together with branch-like, mixed branch-like and fir-like and also with fern like and mixed fir-like and fern-like crystallization. The atypical crystallization was confirmed also in pregnant and non-pregnant animals but only in combination with other type of crystalline patterns. Individual atypical crystallization was noticeable after the second application of preparation of synchronization and only in non-pregnant animals. The atypical crystals had different forms, which are displayed in Fig. 11.

None crystallization was more detected after second application of preparation of synchronization (32.14%) than after first application (10.71%). After insemination this crystallization occurred with higher frequency in pregnant animals than in non-pregnant animals (42.86% vs. 14.29%). Dotted crystallization was noticed in both phases as none crystallization with greater incidence after second application than after first application (42.86% vs. 28.57%). The presence of dotted crystals was only in pregnant animals (28.57% vs. 0%).

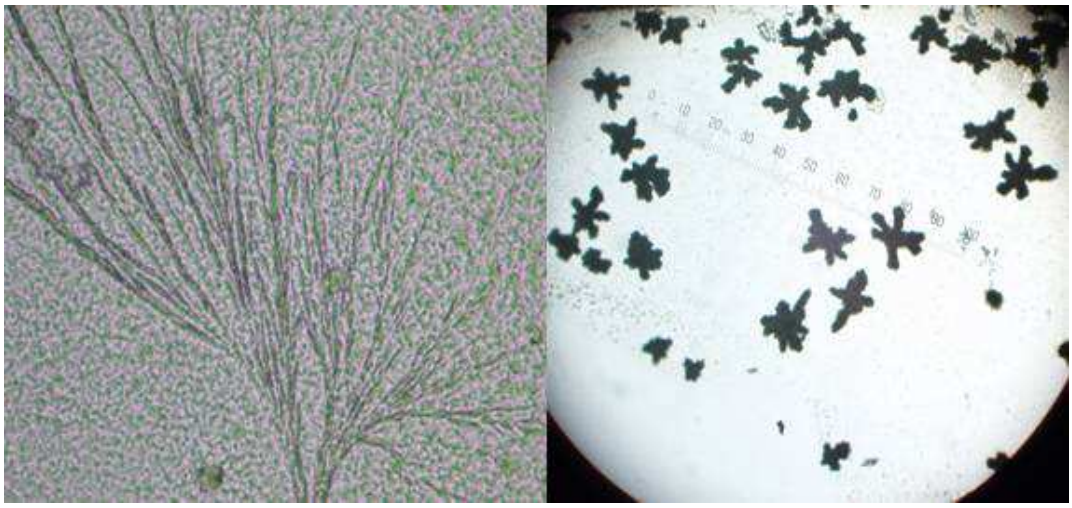

Fig. 11: Examples of atypical crystallization (photo by author)

5.2. The evaluation of individual stages

As for characterization of insemination stage, there were marked only two types of crystallization namely mixed branch-like and fir-like and mixed branch-like, fir-like and fern-like. Higher frequency was noticed in mixed branch-like and fir-like (62.50% vs. 37.50%). The occurrence of mixed branch-like and fir-like crystals grew from the first application of preparation of synchronization till the day of insemination. The artificial insemination influenced occurrence of some crystalline patterns, such as mixed fir-like and fern-like, fern-like and mixed branch-like and fern-like. Thus in time of insemination none most developed crystallization was detected.

In stage after the first application dominated branch-like crystallization (68.75%). Also after the second application highest frequency was noticeable in branch-like crystallization (42.50%). In this stage the first occurrence of fern-like patterns was monitored. Also atypical crystallization was detected for the first time in this phase.

The occurrences of individual general types of crystallization in stages of synchronized estrus are demonstrated in Fig. 12.

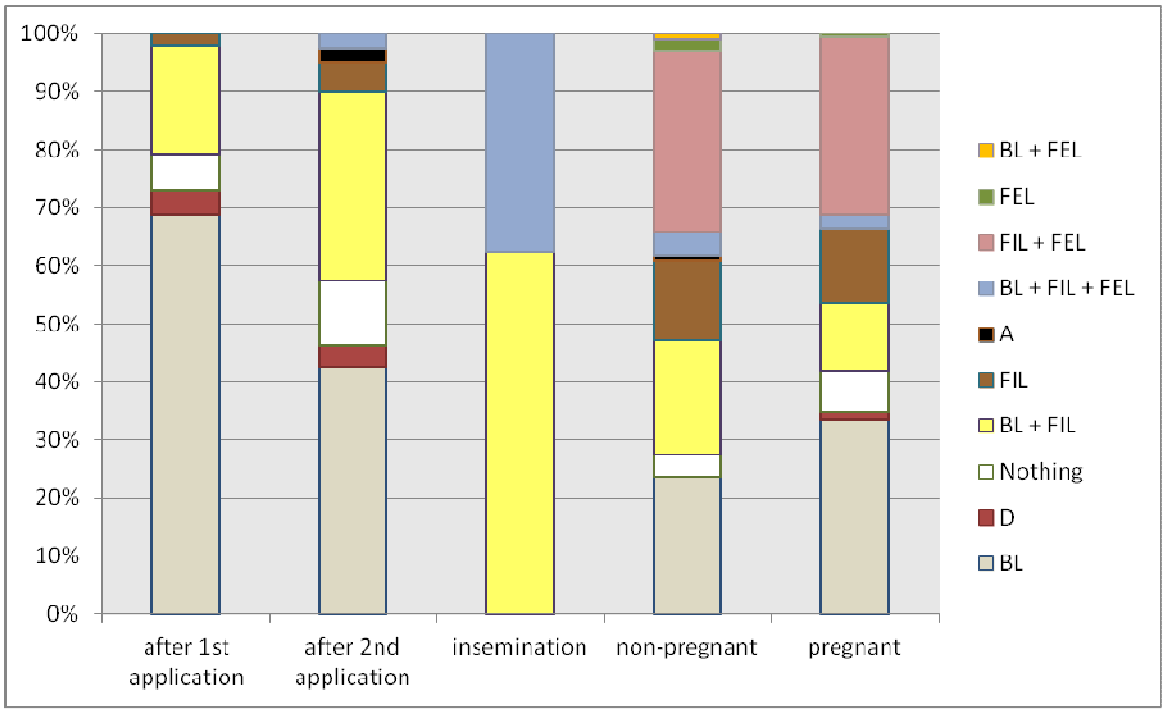

Fig. 12: The appearance of general types of crystallization in different stages of synchronized estrus.

5.2.1. Evaluation of period after insemination

As for pregnancy diagnosis any significant differences were not discovered between individual types of crystallization in pregnant and non-pregnant animals (Kruskal Kruskal-Wallis ANOVA, $p > 0.05$). This conclusion was determined according to results from Fig. 13.

But the differences in distribution of data in pregnant and non-pregnant animals were confirmed (Pearson´s chi-square test: 315,233, DOF = 117, $p < 0.0001$).

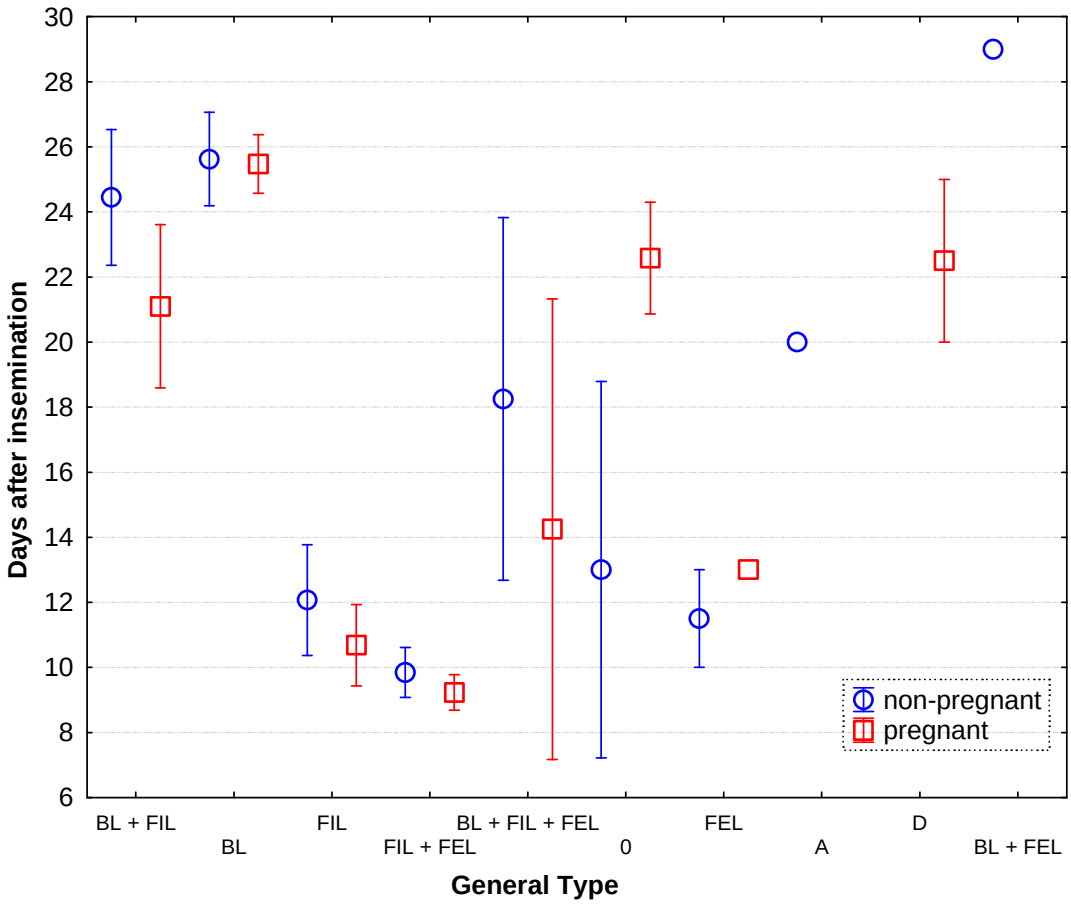

Fig. 13: The graph of occurrence of types of crystallization in pregnant and non-pregnant animals.

5.2.1.1. The evaluation of occurrence of individual types of crystallization

None crystallization occurred from 16th day after insemination mostly with higher frequency in pregnant animals. The biggest difference was seen between the day 26th and 30th (31.25% in pregnant vs. 6.25% in non-pregnant). Dotted crystallization appeared in two cases (16th - 20th day and 26th - 30th day after insemination) and only in pregnant animals. Branch-like crystals were more detected in pregnant animals. The highest difference was marked after 21st day after insemination (24.69% vs. 9.88%). Before this day the contrast between pregnant and non-pregnant animals was not so visible. Mixed branch-like and fir-like dominated in pregnant animals from 16th day after insemination (10% vs. 5%). But from 21st day after insemination this crystallization prevailed in non-pregnant cows (12.50% vs. 7.50%). In the end of research this ratio was equal (12.50%

vs.12.50%). Fir-like patterns mostly predominated in pregnant animals during few days after insemination. After 21st day was observed only in non-pregnant animals (2.78%) and after 26th day after insemination none fir-like crystals were occurred. Mixed fir-like and fern-like crystallization significantly dominated in pregnant animals up to 15th day after insemination. This feature was most visible after 10th day (23.81% in pregnant vs. 13.10% in non-pregnant). After 16th day this crystallization prevailed in non-pregnant animals, but this effect was not important as before (4.76% vs. 2.38%). From 21st day after insemination mixed fir-like and fern-like crystallization was not appeared. Mixed branch-like and fern-like was observed only in non-pregnant animals between the days 26th – 30th after insemination. Fern-like crystallization was detected in interval from day 6th till day 16th. At first fern-like patterns prevailed in non-pregnant cows (33.33% vs. 0%) and later it was balanced (33.33% vs. 33.33%). After 16th day this type was not noticed. During first five days after insemination mixed branch-like, fir-like and fern-like crystallization occurred in pregnant animals with higher frequency (25% vs. 12.50%). Till 16th day this arborization did not occur. From day 16th till day 25th mixed crystallization occurred only in non-pregnant animals (12.50% vs. 0%) and later prevailed in pregnant animals (25% vs. 12.50%). In the end of observation this type of crystallization was not observed.

5.2.1.2. The assessment of individual five-day intervals

The evaluation of post-insemination period after each five days showed the changes in prevailed type of crystallization. From insemination till 16th day after insemination mixed fir-like and fern-like crystallization occurred with the highest frequency and mostly with dominance in pregnant animals (54.17%; 80% vs. 46.67%; 73.33%). Between the days 16th and 20th dominated fir-like crystallization with frequent occurrence in pregnant animals (32% vs. 26.67%). The appearance of mixed fir-like and fern-like crystals visibly decreased (26.67% in non-pregnant animals vs. 8% in pregnant animals). In this period increasing potential of branch-like crystals occurrence was noticeable (from 0% to 28% in pregnant vs. from 0% to 13.33% in non-pregnant). After 21st day prevailed branch-like arborization with higher frequency in pregnant animals (83.33%; 53.85%; 70% vs. 53.33%; 40%; 58.33%) and occurrence of fir-like decreased (6.67% in non-pregnant vs. 0% in pregnant). None mixed fir-like and fern-like crystals were detected from 21st day after insemination. In Tab. 2 are displayed observed intervals from 16th day after

insemination with occurrences of different types of crystallization in pregnant and non-pregnant animals.

Tab. 2: The changes in dominance of crystallization in pregnant (P) and non-pregnant (N) animals from 16th day after insemination (columnar frequencies from contingency tables).

Intervals →	**Day 16th - 20th**		**Day 21st - 25th**		**Day 26th - 30th**		**Day 31st – 34th**	
General types ↓	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**
0	12%	6.67%	4.17%	0%	19.23%	6.67%	5%	0%
D	4%	0%	0%	0%	3.85%	0%	0%	0%
BL	28%	13.33%	**83.33%**	53.33%	**53.85%**	40%	**70%**	58.33%
BL + FIL	16%	13.33%	12.50%	33.33%	15.38%	40%	25%	41.67%
FIL	**32%**	26.67%	0%	6.67%	0%	0%	0%	0%
BL + FEL	0%	0%	0%	0%	0%	6.67%	0%	0%
FEL	0%	0%	0%	0%	0%	0%	0%	0%
BL+FIL+FEL	0%	12.50%	0%	12.50%	25%	12.50%	0%	0%
FIL + FEL	8%	26.67%	0%	0%	0%	0%	0%	0%
A	0%	6.67%	0%	0%	0%	0%	0%	0%

5.3. The additional characteristics of crystals

The thickness of crystals was characterized together with type of crystallization. Whole types of crystallization were represented by all levels of thickness. The occurrence of the highest values of thickness was noticeable in many forms. Maximal thickness was detected in the whole surface of crystals and also in the terminal parts of crystals (Fig. 14). In some crystals base started on the lowest level of thickness and end parts were presented by the highest value.

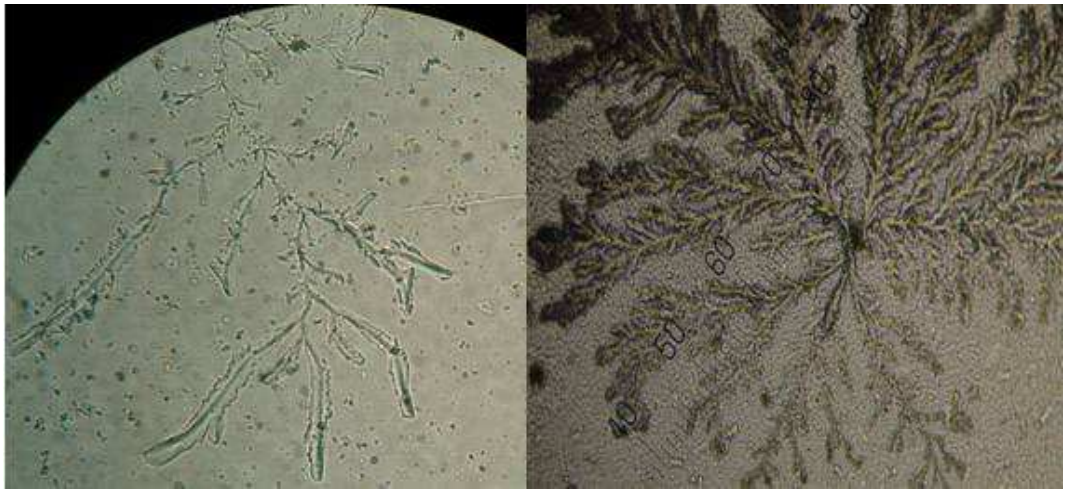

Fig. 14: thickness of terminal parts of crystals (photo by author)

The density of crystals was influenced by sample quality (Kruskal-Wallis test: H (3, N= 408) =123.2143 $p < 0.00001$). The samples of good quality (OK) had significantly higher (multiple comparison test, $p < 0.05$) densities than samples with contamination or thin smear (Fig.15). The significant difference ($p > 0.05$) was not detected between the density in contaminated samples and thin smears. The density of crystals was also compared with thickness of crystals. The weak correlation was found between density and thickness ($r = 0.3151$, $p < 0.00001$). The statistically significant difference (Kruskal-Wallis test: H (3,408) = 13.6003, $p < 0.0035$) was confirmed in samples with good quality and samples with thin smear, but contamination in samples did not have statistically significant influence on thickness of crystals (multiple comparison test, $p > 0.05$).

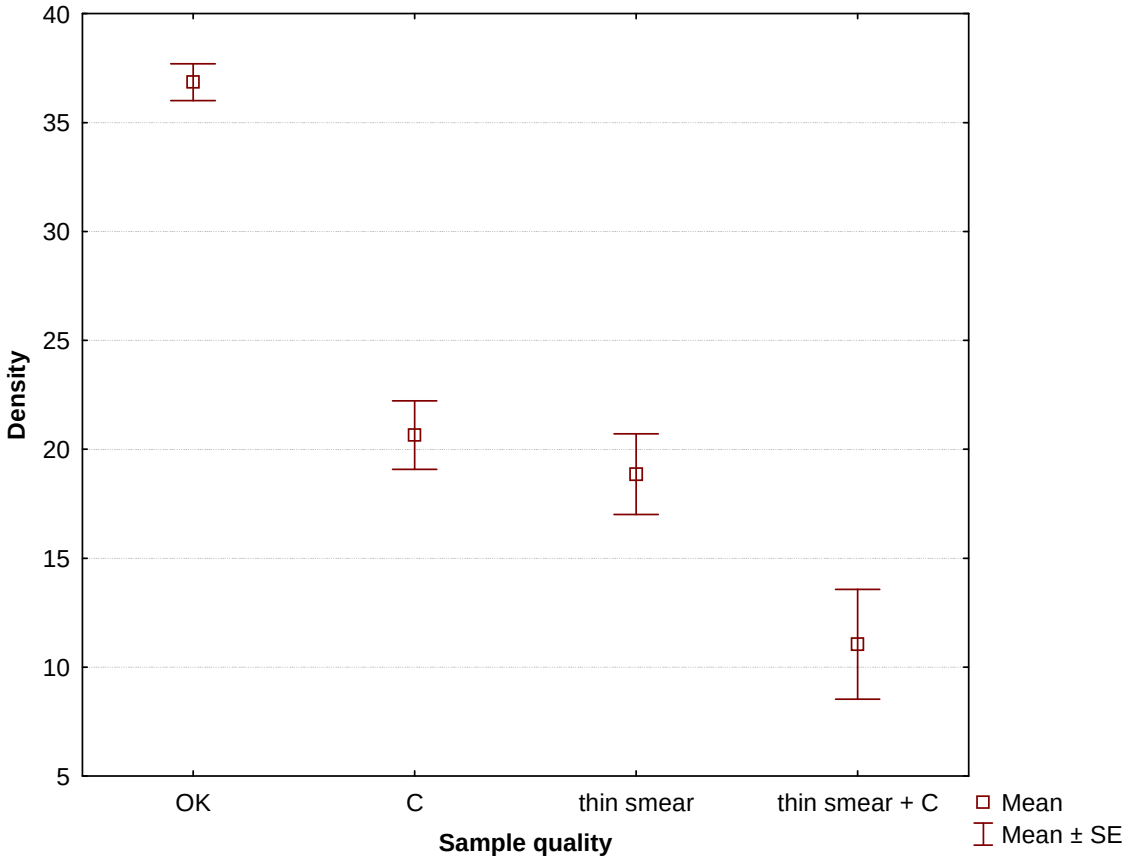

Fig. 15: The graph of influence of sample quality on density of crystals

The connection between density and general types of crystallization was verified (Kruskal-Wallis test: H (9, N= 408) = 151.8951, $p < 0.0001$). This finding is demonstrated in Fig. 16. Significant differences between branch-like crystallization and mixed branch-like and fir-like, mixed branch-like, fir-like and fern-like and mixed fir-like and fern-like were confirmed according to multiple comparison test ($p < 0.05$). The branch-like crystals occurred in lower density. Fir-like arborisation was verifiably different from dotted crystallization and significant difference was checked between dotted crystalline patterns and mixed branch-like and fir-like, mixed branch-like, fir-like and fern-like and mixed fir-like and fern-like. Dotted crystallization appeared in lower density.

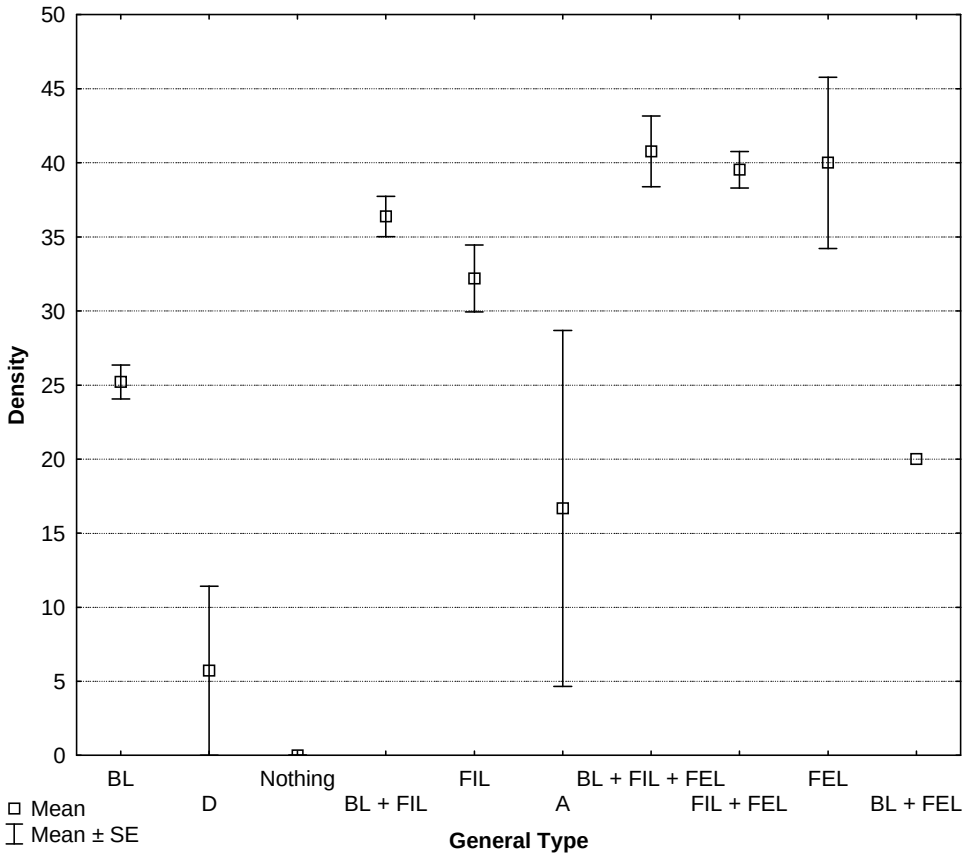

Fig. 16: The graph of relationship between density of crystals and general types of crystallization.

5.4. The coloring of samples by Giemsa stain

The staining of crystals by Giemsa was unsuccessful, because this process did not improve the better searching of crystals. On the contrary stained crystals were less noticeable or generally damaged and the contaminant and other side materials were colored primarily.

6. Discussions

Compared with other studies focused on crystallization of different body fluids we discovered some similarity and also differences. These features were observed from methodology to results of other studies.

The methodology of presented study was inspired by study of Haberová (2010). The samples of saliva were obtained by plastic coffee stirrer. The sampling by coffee stirrer is not complicated but there is possibility of damaging the stirrer and consequently swallowing. Also plastic coffee stirrer can influence the quality of samples and the structure of crystallization. Other unsuitable tool to obtain saliva is frosted glass slide put on tongue, which was used in bitches by Pardo-Carmona (2010). In this case the glass can injure tongue or animal can destroy the glass slide and consequently swallow fragments. But in this study a finger gloved was used for obtaining saliva. Consequently a finger gloved was impressed on the glass slide. This method could be used in cattle.

Evaluation of samples was done according to Haberová (2010). From study of camels the scoring system was taken and applied on saliva crystallization in cattle. But later this system of classification was used only as model of possible similar features of defined type of crystallization. Thus the arborization in cattle differed from crystallization in camels in all cases of observation.

Monitoring of saliva crystallization or crystallization of other body fluids was made mainly by microscope at magnification 400x. But Pardo-Carmona et al. (2010) examined crystals at magnification 100x. But this observation could cause overlooking of some details in created crystals. According to study of saliva crystallization in cattle the magnification 100x is considered to be more suitable for assessment of density and occurrence of crystalline patterns.

Classification of the crystallization was mostly done subjectively; this means that one author of research decided the type or level of crystallization (Habeorvá, 2010; Pardo-Carmona, 2010). But in the study of Alliston et al. (1958) the determination of arborization was done by two authors independently. From their observations the median was assessed and consequently the type of crystallization was determined.

The classification of combinations of saliva crystallization in cattle was difficult due to variations of basic types.

In any study the shape of crystals was not described in details as in the research of camel´s and cow´s saliva. Apart from the study of Golding and Brennan (1989) where were mentioned abundance of crystals and spaces between branches.

Some similarity in patterns of saliva crystallization in cattle was discovered in other types of arborization of different body fluids for example in saliva and cervical mucus in bitches (Pardo-Carmona, 2010; England & Allen, 1989), cervical mucus in bovine (Noonan et al., 1975), human cervical mucus (Zondek & Rozin, 1954), cervical mucus in pigs (Betteridge & Raeside, 1962) and human tears (Golding & Brennan, 1989). The atypical crystallization of cervical mucus was described in Rob and Stehlík (1983). Significant similarity of atypical crystallization was noticeable between saliva and cervical mucus in cattle. Some of the atypical crystals in saliva were identical to atypical crystals in cervical mucus during metabolic disorders and inflammatory processes. There is a consideration that the atypical crystallization was caused by bad quality of smears or by some metabolic or reproductive diseases, which was not detected and confirmed by farmer of CULS Estate Lány – Ruda. The branch-like crystallization in cows was represented by different variants. The base of branch-like crystals was similar in cows and camels, but the differences were noticed in size of crystals and in connection of individual side branches to the main branch. The comparison of branch-like crystallization in cows and camels is shown in Fig. 17.

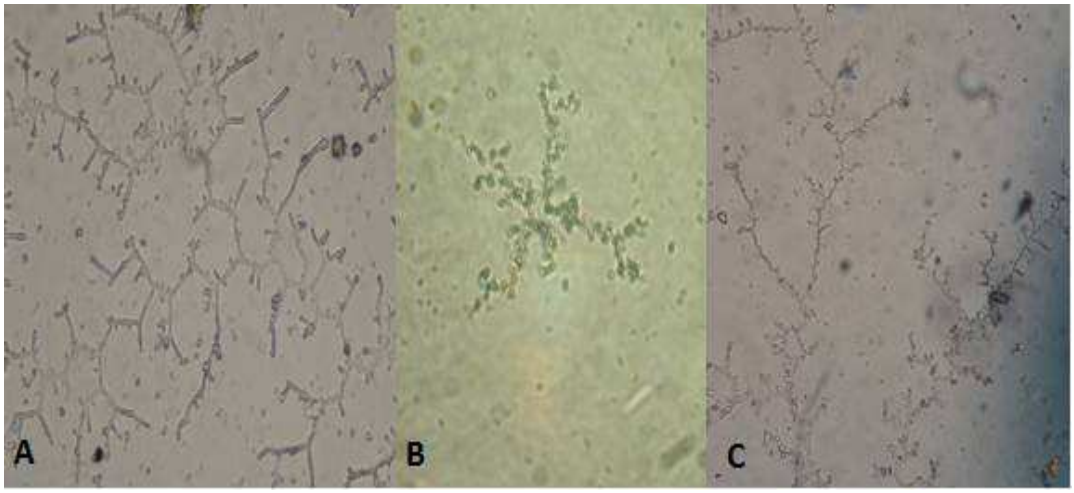

Fig. 17: Comparison of BL crystallization in camels and cows

A: BL crystals in camels (photo by Haberová, 2010)

B: BL crystals in cows (photo by author)

C: BL crystals in cows (photo by author)

The frayed crystals were the other feature, which was recorded during examination. It was detected mainly in mixed branch-like and fir-like crystallization or in fir-like crystallization. The fraying was visible in whole crystals or only in terminal parts of branches. This effect was not considered as a consequence of any disease or disorder, because the occurrence of frayed crystals was noticeable with high frequency. This phenomenon is shown in Fig. 18.

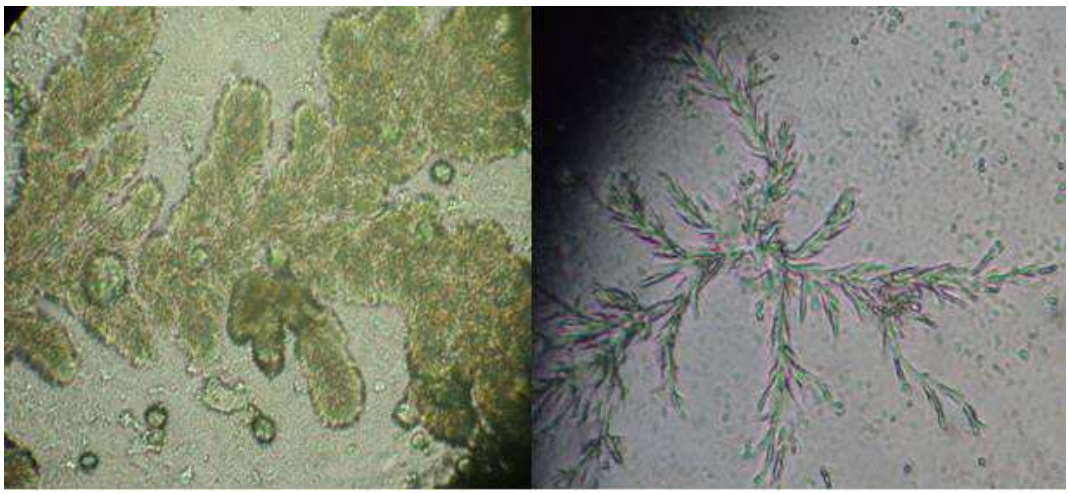

Fig. 18: The fraying effect of crystals (photo by author)

The low occurrence or less developed crystallization could be influenced by bad sampling and subsequent smearing. Because some crystals could be destroy by strong movements of coffee stirrer. Also the contaminants in samples could influenced occurrence of less developed or none crystallization. In the contaminated samples were found rests of feeds.

The connection between type of saliva crystallization and stage of reproductive cycle was not discovered, it could be due to synchronization of estrus. Because tested animals did not pass through the whole reproductive cycle and it could be concluded, that saliva crystallization, scored by mentioned system, could not be used in practice as tool for heat detection.

The changes in crystallization after insemination were described. The level of crystallization decreased during the period of next estrus and the lowest level of crystallization dominated during following days. These results were noticed only in pregnant animals. It was considered, that sampling of cattle saliva and the changes in saliva crystallization after insemination could be implemented to practice. Because of saliva

sampling is simple and cheap method as well as observing of crystallization. According to the results, changes in arborization after insemination in time of next estrus can distinguish cycling and non-cycling animals or perhaps pregnant animals and cycling animals. But only 8 animals were included in research. There is a possibility of distorted results. The further and more detailed examination of crystals in cattle saliva is needed, especially crystals occurring in cycling and non-cycling animals.

The localization of crystals was not evaluated, but according to subjective observation was concluded, that the biggest presence of crystals was in the thicker layer of saliva, near very small contamination, bubbles and edge of a glass slide. In study of Alliston et al. (1958) the occurrence of crystals around bubbles was categorized to scoring system.

The occurrence of individual types of crystallization was compared only with studies focused on saliva crystallization in animals, namely camels (Haberová, 2010) and bitches (Pardo-Carmona et al., 2010). The bigger similarity was discovered in study of camels, because there were also confirmed all types of crystallization and one of the most common arborization was branch-like. In case of bitches most developed crystallization occurred a few days around optimal time of mating contrary in cows most developed arborization appeared after insemination.

The quality of samples and monitoring of crystals was not influenced by storage of samples. The smears can be preserved for a few months at room temperature. The glass slides were closed in plastic or paper transporting box against destruction or contamination. The negative influence of preservation on crystallization was verified by control observation of selected glass slides after a few months.

None studies were discovered to compare method of saliva staining by Giemsa. Unsuccessful samples coloring by Giemsa stain could be caused by wrong method of staining. The saliva crystallization could be damaged by higher concentration of Giemsa or long time of action. The process of coloring was not repeated and other concentrations and times of influence were not tested.

7. Conclusion

Completely were evaluated 408 samples of cattle saliva. The saliva arborization and its changes in type of crystallization were confirmed in all of examined animals. Other signs of changes in saliva arborization were marked in size of crystals, level of development, density of individual crystals, thickness and entirety of crystals and frequency of occurrence of crystallization in one sample.

Three most common types of crystallization were detected during whole period of observation, namely branch-like, mixed branch-like and fir-like, mixed fit-like and fern-like. Besides common types of arborization the atypical crystallization was noticed. It was detected together with branch-like, mixed branch-like and fir-like and also with fern like and mixed fir-like and fern-like crystallization. The atypical crystallization was also presented in pregnant and non-pregnant animals as a supplement of other types of crystallization.

The branch-like crystallization dominated after both applications of preparation of synchronization. Only two types of arborization were marked in time of artificial insemination, such as mixed branch-like and fir-like and mixed branch-like, fir-like and fern-like. The higher frequency was noticeable in case of mixed branch-like and fir-like.

The thickness, entirety and density were evaluated as supplemental characteristics of crystals. The statistical conclusiveness was confirmed only in case of density of crystals in relationship with sample quality. This means that samples with good quality were represented by higher densities of crystals than contaminated samples or samples with thin smear of saliva. The general types of crystallization occurred in different levels of density. The results showed that less developed crystallization (dotted, branch-like) appeared in lower densities than more developed crystals (fir-like, mixed branch-like and fir-like, mixed branch-like, fir-like and fern like, mixed fir-like and fern-like).

The changes of crystallization were detected after insemination together with difference between pregnant and non-pregnant animals during a few days after insemination. The fir-like and fern-like crystallization dominated from insemination till 16^{th} day after insemination. The fir-like crystallization is less developed type and prevailed between the days 16^{th} – 20^{th}. The lowest level of these crystallizations, branch-like,

dominated after 21st day. These highest occurrences were detected only in pregnant animals.

The Giemsa stain, used by mentioned process, was not considered as a tool for better searching of crystals. The connection between type of crystallization and reproductive cycle in cattle was not discovered in animals, which were included into synchronized estrus programme. I cannot recommend the usage of this method in practice yet, because the values of crystallization in my study were not statistically significant. According to the results, the recognition of phase of reproductive cycle using saliva crystallization, evaluated by mentioned score, is not possible.

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APPENDICES

List of the Appendices:

APPENDIX 1

Table used for evaluation of crystallization

DATE	NUMBER OF COW	TYPE OF CRYSTALLIZATION	THICKNESS OF CRYSTALS	ENTIRETY OF CRYSTALS	DENSITY OF CRYSTALS	SAMPLE QUALITY	NOTES	LIST OF ABBREVIATIONS
	1.							**O**-none
	2.							**BL**-branch-like
	3.							**BL+FIL**-mix branch-like and fir-like
	4.							**BL+FEL**-mix branch-like - fern-like
	5.							**FIL**-fir-like
	6.							**FIL+FEL**-mix fir like and fern-like
	7.							**FEL**-fern-like
	8.							**BL+FIL+FEL** – mixed branch-like, fir-like and fern-like
	1.							**D**-dotted
	2.							**A** - atypical
	3.							
	4.							
	5.							
	6.							
	7.							
	8.							