ARTIFICIAL INSEMINATION WITH CHILLED SEMEN IN DOGS

Zabret, Ajda

UNIVERSITY OF ZAGREB

FACULTY OF VETERINARY MEDICINE

Ajda Zabret

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University of Zagreb
Faculty of Veterinary Medicine
Clinic for Obstetrics and Reproduction

Head of the Clinic: prof. Marko Samardžija

Mentor: Prof. Martina Lojkić, PhD, DVM
Assoc. Prof. Branimira Špoljarić, PhD, DVM

Members of Committee for the Diploma thesis defense:
1. prof. Juraj Grizelj, PhD, DVM
2. Ivan Butković, PhD, DVM
3. prof. Martina Lojkić, PhD, DVM
4. prof. Nikica Prvanović Babić, PhD, DVM

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FIGURE LIST

TABLE LIST

ABBREVIATIONS' LIST

AI = artificial insemination

CS = chilled semen

LH = luteinizing hormone

FSH= follicle stimulating hormone

GnRH = gonadotropin-releasing hormone

CL = corpus luteum

EIU = endoscopic-assisted transcervical insemination

TC = transcervical insemination

SIU = surgical implant

IV = intravenous

CASA = computer-assisted sperm analysis

INDEX

1. INTRODUCTION

Artificial insemination (AI) is imitation of natural act of mating, where semen is collected from male animals and introduced (inseminated) into female reproductive tract during her fertile period, so fertilization can occur. The advantages of artificial insemination are genetic preservation, prevention of spreading certain sexually transmitted diseases by avoiding direct contact between male and female and reduction of inbreeding by allowing for greater pool of studs to be selected from all over the world. Also, it reduces stress in animals caused by long distance transport; it gives opportunity to evaluate semen prior to AI and early detection of male reproductive pathologies. The potential weakness of artificial insemination is possible induction of physical or psychological trauma during AI process, so specialized equipment and technical expertise is needed. Potential overuse of male dogs and lower pregnancy rates can also be a weak point of artificial insemination.

Artificial insemination can be performed with fresh, chilled, and frozen-thawed semen. To be successful, it requires careful assessment of optimal breeding time of the female and semen collection, evaluation, and preparation of male. Also, proper insemination technique must be applied to achieve good pregnancy rate and litter size.

This is a review paper on using chilled semen for artificial insemination in dogs, including up-to-date information about semen collection and semen quality assessment, preparation of dog chilled semen for AI, detecting the optimal timing for insemination and methods of AI in dogs with chilled semen.

2. LITERATURE REVIEW

2.1 INDICATION FOR AI WITH CHILLED SEMEN

Main indications for artificial insemination in dogs are due to medical and breeding-management reasons. The advantage of AI is reducing physical distance and stress otherwise caused by transportation of animals, use of genetically valuable stud dog semen all over the world and reduce the inbreeding. It is also important in cases in which some physical or behavioral abnormalities in male or female dogs could prevent natural mating (PAYAN-CARREIRA et al., 2011). The use of chilled semen is recommended when the semen needs to be transported over short distances, for instance if the semen sample needs to be transported to the female dog within 48 hours after semen collection. In general, within European union, semen can be delivered to most European countries within 24 hours. The procedure is easier and less expensive than semen cryopreservation (RIJSSELAERE et al., 2011; LOJKIĆ et al., 2022).

2.2 ANATOMY OF FEMALE REPRODUCTIVE ORGANS

The genital organs of bitch include paired ovaries, uterine tubes or oviducts, the bi-cornuate uterus with short uterine body, cervix and the vagina. The role of ovaries is to produce female gametes and hormones. Paired oviducts capture the ova which are released from ovaries and transport them to the uterus, where the fertilized ova are preserved. Vagina serves as a copulatory organ, and together with vestibule, as birth canal and passage for urinary excretion (KÖNIG and LIEBICH, 2004).

Ovaries are located in lumbar region, caudal to the kidneys. They are ellipsoidal in shape and their surface is rendered characterized by large follicles and corpora lutea. They measure approximately 1-1.5 cm in bitch. On the cross-section structure of the ovaries consists of looser, vascular zone in the center, called medulla (*zona medullaris seu vasculosa*) and the surrounding denser shell, the parenchymatous zone (*zona parenchymatosa*). The parenchymatous zone is lined by albugineous tunic, directly below the peritoneum. The medulla consists of blood vessels, nerves, lymphatics, connective tissue and smooth muscle fibers, and parenchymatous zone contains follicles and corpora lutea in different stages of development and regression (KÖNIG and LIEBICH, 2004).

Uterine tubes or also termed oviduct or salpinx are paired organ, which receive and transport oocytes to the uterus. Function of oviducts is also the transport the sperm upwards and is a place where fertilization occurs. Each tube is suspended by the mesosalpinx and connects the peritoneal cavity with the uterine cavity and external environment. The ovarian part of the uterine tube is termed infundibulum and it receives the oocytes after ovulation. The edges of infundibulum are bordered by numbers of processes, called fimbria. Inside of the infundibulum is marked by folds, which converge to border a small opening in the funnel, called abdominal ostium. Abdominal ostium leads to ampulla, a place where fertilization occurs. Uterine tube opens into the uterine horns through uterine rostrum which marks the uterotubal junction (KÖNIG and LIEBICH, 2004).

Uterus in carnivores lies mainly dorsally to the small intestines, it consists of short cervix and body from which two horns extend and connect to ovaries. Uterine wall consists of three layers, mucosal (endometrium), muscular (myometrium) and serosa layer (perimetrium). Endometrium lines the lumen of the uterus and its thickness depends on the stage of estrus

cycle. Myometrium underlies the endometrium, and consists of two layers, external longitudinal and internal circular layer, which are separated by highly vascularized connective tissue. Uterus is surrounded and covered by the serous membrane called perimetrium, which contains many blood vessels and nerve fibers (KÖNIG and LIEBICH, 2004).

Cervix is the lower part of the uterus, which connects the uterus and vagina. The lumen of the cervix is called cervical canal and is formed by mucosal folds, which are arranged longitudinally in bitches. The cervical canal opens cranially into a body of the uterus and the internal uterine ostium and caudally into the vagina at the external uterine ostium. Cervical mucosa secretes mucus which forms a plug that helps close the cervix; mucus is secreted during estrus, and plug is expelled before parturition. It has an important role in allowing the passage of sperm into the uterus (KÖNIG and LIEBICH, 2004). The cervical lumen extends cranially from cervical opening, which in most cases faces the floor of the cervix (MASON, 2018).

Vagina extends from the external opening of the uterus to the entrance of urethra and its quite long in bitch. In dogs, vaginal epithelium responds to hormonal changes, which can be helpful in staging the cycle and estimating the optimal breeding time, by examining vaginal smears. Caudally to the cervix in the dorsal wall of vagina, there are 3 dorsomedial folds which consists of 3 components: caudal, middle and cranial tubercles. Cranial vagina ends at the cervix, which protrudes caudally into vagina, and this creates a cavity between the opening of the cervix and cranial part of vagina, and this is called fornix (MASON, 2018).

Vulva is the external part of the female genitalia and the entrance to the vestibule. Its structure is formed by two labia that meet at the dorsal and ventral commissures, surrounding the vertical vulvar opening. In the ventral commissure the clitoris lies, and the urethral opening. Vestibule is quite short and is terminated at the cingulum, a narrowing, which has its function during a copulatory tie, to lock around bulbs penis to maintain erection. From cingulum vagina extends forward parallel to the lumbar spine, is quite long and it extends to the level of cervix (MASON, 2018).

Female genital organs are attached by paired double folds of peritoneum, left and right broad ligaments of the uterus called *ligament latum uteri*, which are bilateral sheets that suspend the ovaries, uterine tube and uterus from abdominal roof and pelvic walls. Broad ligament can be

divided into three parts, mesovarium, mesosalpinx, mesometrium, based on the organs it suspends. Cranial part of the broad ligament is mesovarium, which attaches the ovary to the dorsolateral region of abdominal wall, and it contains ovarian artery and vein. Mesosalpinx extends laterally from the mesovarium, dividing the mesovarium into proximal mesovarium which extends from the body wall to the mesosalpinx and distal part, which extends from the mesosalpinx to the ovary. Mesometrium is the largest part of the broad ligament, which attaches to the uterus and the cranial part of the vagina. There are also other ligaments, like suspensory ligament of the ovary, proper ligament of the ovary and round ligament of the uterus, which help in keeping genital structures in the right place (KÖNIG and LIEBICH, 2004).

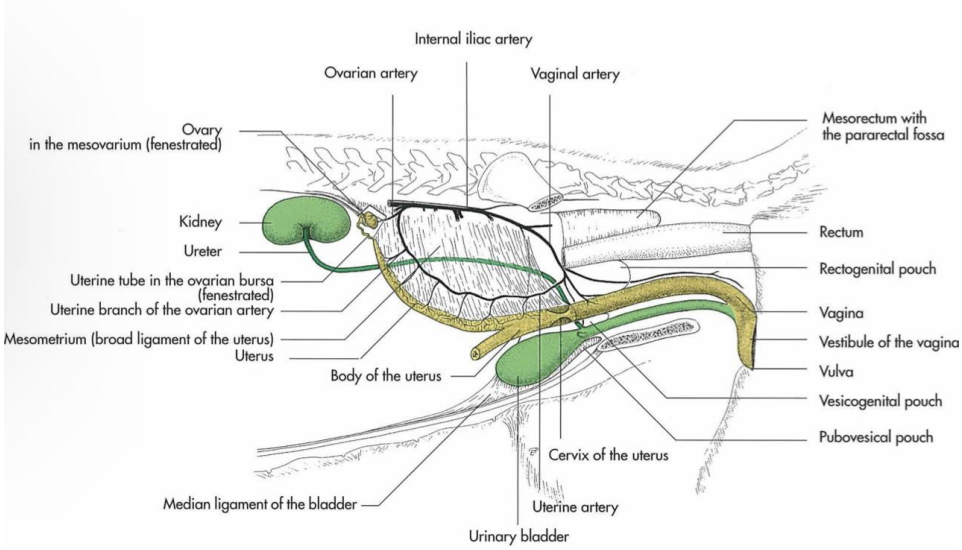

Figure 1. Female genital organs of the dog, schematic (KÖNIG and LIEBICH, 2004).

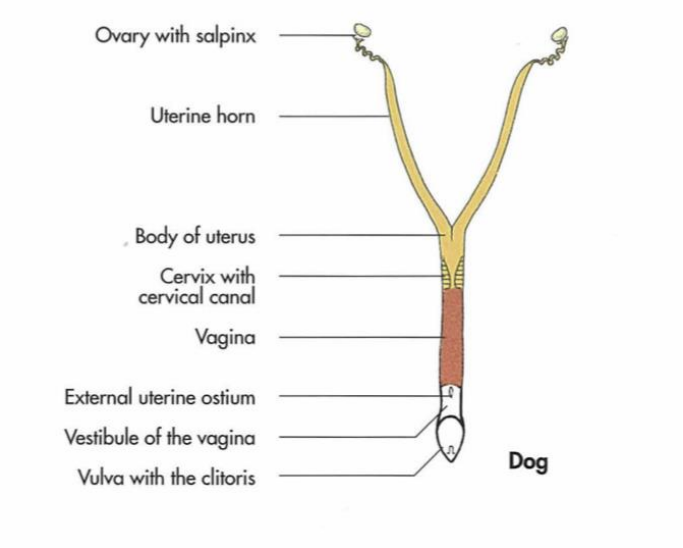

Figure 2. Female genital organs of the dog, schematic (KÖNIG and LIEBICH, 2004).

2.3 PUBERTY, MATURITY AND SENESCENCE IN BITCH

Puberty in bitch usually occurs between 6 and 15 months of age, in larger breeds mostly at 18 - 20 months of age and is not dependent on the day length, exception only being Basenji which usually cycle in autumn (ROOT KUSTRITZ, 2010). Puberty is also related to size and weight of the bitch, and it occurs when bitch has reached approximately 85% of adult weight. Bitches of smaller breed tend to have their first cycle at earlier age than those of large breeds. In pubertal estrus the circulating hormone levels are usually lower and fluctuating, which can result in absence or incomplete ovulation and the bitch may not show the standing estrus (CHAKRABORTY et al., 1980; WILDT et al., 1981). Size of the litter is breed-related, and it increases until 3 years of age and decreases after 7 years of age. According to HOLLINSHEAD and HANLON (2017) smaller breeds have significantly smaller litters than medium, large and giant breeds, while for each advancing year of age, litter size decrease by 0.13 pups per litter. Senescence is considered not to occur in dog. Bitches cycle, and in case of mating they can become pregnant all their life, even though their fertility decreases with age.

2.4 ESTROUS CYCLE IN BITCH

Female dogs are monoestrous, typically non-seasonal, polytocous, spontaneous ovulators and have a spontaneous luteal phase of long duration, approximately 65 days, followed by an obligate anestrus before the next 2–3 week “heat” (proestrus-estrus). The resulting interestrus intervals of 5–12 months are very variable between bitches, commonly 6–7 months (CONCANNON, 2011; ROOT KUSTRITZ, 2012).

The estrus cycle consists of the following stages: proestrus, estrus, meteoestrus (diestrus) and anoestrus.

Proestrus is considered to start when serosanguineous discharge has been noticed, together with vulval reddening and enlargement. Discharge usually marks the first day of proestrus. Proestrus can last 3-27 days but on average around 9 days. In proestrus bitch is attractive to male but she is not ready to mate. Some behavioral changes that can be noticed include restlessness, urine marking and increased attractiveness to dogs. In proestrus, there are high levels of estradiol, which stimulates growth and activity of glandular epithelium and promote mucosal vascularity and edema. Proestrus is also associated with follicle development, which is stimulated by LH and FSH, which are secreted from anterior pituitary, under the influence of GnRH. The surge in plasma LH concentration always occurs automatically after a certain level of follicular activity and estradiol output, and due to LH peak, spontaneous ovulation follows. To stimulate follicle growth and estradiol secretion, FSH is needed, but the circulating FSH does not increase in same amount as LH, because inhibin is secreted from follicles, which selectively inhibit FSH secretion. There is a rise of progesterone secretion by the pre-ovulatory follicles, which have an important role in triggering ovulation and standing estrus. Progesterone levels are still low until late proestrus or early estrus (JEFFCOATE, 1998).

Oestrus is a phase in which ovulation occurs and is characterized by bitch being ready to mate and accept the male. In some females it can last only for 2-3 days while in others even up to 21 days, but with average of 9 days (ROOT KUSTRITZ, 2012). Vulva is softer but still enlarged and discharge from vulva is lighter in color. Estradiol drops to basal concentration and progesterone levels increase rapidly. Preovulatory LH peak occurs in this phase and approximately 2-3 days after LH peak ovulation occurs (JEFFCOATE, 1998). Bitches can

have multiple ovulations and most ovulations occur within 30-48 hours after LH peak, but some follicles may not ovulate until as late as 96 hours after. The duration of ovulation can vary within 24-96 hours. Oocytes are ovulated as primary oocytes and they must undergo a further maturation before fertilization. This maturation can last 2-3 days, which results in oocytes being capable of fertilization, usually in the second half of estrus, and this phase is then termed fertile period. Fertile period is quite long in bitches and can range from 4 to 7 days post LH peak. Long term intrauterine sperm survival (up to 5 days) and time-spread of ovulation and oocytes maturation are factors responsible for long fertile period (JEFFCOATE, 1998).

Next phase is **metoestrus** or also called diestrus and is a phase in which bitch is not receptive to mating anymore. It usually occurs 6-8 days after onset of estrus or 8-10 days after preovulatory LH peak and last approximately 2 months. Vulva decreases in size, discharge from vulva can be still present but is more clear, purulent, or also hemorrhagic. There are no discrete endocrine changes in this phase, only plasma progesterone concentrations, which increase progressively from onset of estrus. This phase is dominated by progesterone, and entire gestation period fits into metoestrus. In both pregnant and non-pregnant bitches, progesterone is secreted form CL. Early stages of embryo development are also included into phase of metoestrus. After the onset of metoestrus, plasma progesterone concentration continues to increase reaching the peak value 90-270 nmol/L (30-90 ng/ml), about 30 days after the LH peak, and it slowly decrease to about 30 nmol/L (10 ng/ml) by day 60 after the LH peak. Progesterone concentration should stay within a range 30-90 nmol/L (10-30 ng/ml) for approximately 2 months after estrus, and in case it does not, it indicates an anovulatory cycle or some other abnormalities in luteal function. Plasma progesterone does not differ between pregnant and non-pregnant bitches by day 60, but later, in pregnant bitches, plasma progesterone concentration falls abruptly before parturition, while in non-pregnant bitches progesterone levels fall gradually to 2-9 nmol/L (1-3 ng/ml), over the next 30-60 days, due to absence of luteolytic mechanism. High plasma prolactin concentrations can be seen from 30-65 days after LH peak and both pregnant and non-pregnant bitches and continue to be high especially in suckling bitches, from days 60-69 (JEFFCOATE, 1998).

Last phase of the cycle is **anoestrus** which is defined as an interval between parturition and proestrus and would normally include lactation. In non-pregnant animals the anoestrus is defined as interval between end of luteal phase and onset of proestrus. It lasts approximately 4

months. Estradiol is low until late anestrus when it starts to rise again, progesterone levels are low and LH pulses increase in late anestrus. Corpus luteum is in state of regression (JEFFCOATE, 1998).

Hormonal regulation of estrus cycle

Estrus cycle in bitch is regulated by hypothalamus-pituitary gland-ovaries axis, which secrete different hormones for estrus cycle to be completed. Hypothalamus is located on the base of the brain and is responsible for production and release of gonadotropin releasing hormone (GnRH). Anterior pituitary not only has a direct effect on ovarian function, but also ovary influences hypothalamus and anterior pituitary, by negative/positive feedback of estradiol from maturing follicle, and negative feedback of progesterone form corpus luteum. The main hormones involved in sexual cycle of the bitch are pituitary hormones, follicle stimulating hormone (FSH), luteinizing hormone (LH) and the steroids, estrogen and progesterone, which are secreted from ovaries. Prolactin and growth hormone (GH) are also part of the sexual cycle, but mainly in relation to pregnancy and parturition.

GnRH is produced by hypothalamus and the release of GnRH has direct action on front lobe of pituitary gland and further production and release of gonadotropins, FSH and LH. It also regulates concentration of ovarian hormones via feedback mechanism (ROBINSON and NOAKES, 2019). Release of FSH is controlled by GnRH, and it stimulates growth and maturation of ovarian follicles. LH is responsible for maturation of follicles and oocytes, ovulation and formation of corpus luteum. It is produced and released from anterior pituitary gland. Secretion of FSH and LH is controlled by two functionally separate but still connected systems: **episodic/tonic system**, which is responsible for the continuous basal secretion of gonadotropin which stimulates growth of germinal and endocrine components of the ovary; the second is the **surge system**, which controls the short-lived secretion of gonadotropin, especially LH, and is responsible for ovulation (RAHE et al., 1980). Release of inhibin end estradiol from follicles will suppress the FSH secretion (BAIRD et al., 1991). The decline in the rate of increase in estradiol in late proestrus cause the surge release of LH, which results from increased peripheral estradiol clearance as well as from follicles reaching terminal maturation (CONCANNON et al., 2009).

Oestrogen is an ovarian steroid hormone which is secreted from granulosa cells of developing follicles and is responsible for normal sexual and estrus behavior and development. Some internal changes induced by estrogen can include cornification, oedema and elongation of vagina, hyperemia and elongation of uterine horns, enlargement of oviducts and proliferation of fibrillated end of oviduct. External changes noticed due to high estrogen can include vaginal discharge of uterine blood and enlargement, oedema and hyperemia of vulva (CONCANNON and BATTISTA, 1986). Levels of estrogen (estradiol and oestrone) are already elevated at the onset of proestrus and increase to reach peak levels of 50-120 pg/ml, 1-2 days prior to LH surge, and then rapidly falls during estrus (CONCANNON et al., 1975).

Progesterone is also an ovarian steroid hormone, which has a major role in control the estrus cycle and maintenance of pregnancy in bitch. It has a very important role in the inhibition of the tonic mode of LH secretion (GOODMAN and KARSCH, 1980), by reducing LH pulse frequency. The level of progesterone is basal (< 0.5nmol/L) until the end of proestrus, when the follicles change from producing estradiol to producing progesterone, shortly before LH peak. The long preovulatory progesterone production is unique for bitch. During LH peak, progesterone is usually around 6-9 nmol/L, and ovulation occurs approximately 1-2 days later, at progesterone level of 12-24 nmol/L. After that, progesterone rises rapidly to around 150 nmol/L and slowly decreases during following 2-3 months (LINDE FORSBERG, 2001). The initial increases in progesterone serve to facilitate the rapidity and magnitude of the LH surge (CONCANNON, 2011).

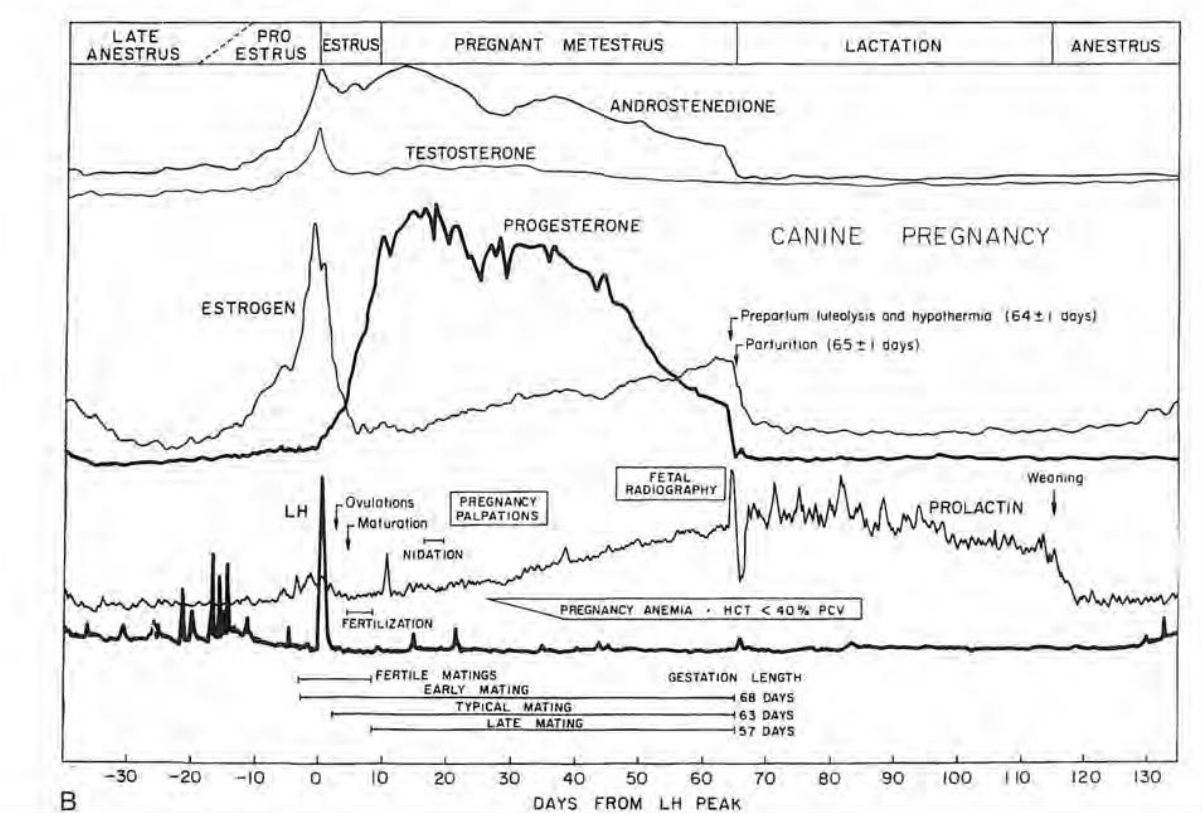

Figure 3. Endocrine hormonal changes in plasma concentration during different phases of estrus cycle in bitch (JOHNSTON et al., 2001).

2.5 ANATOMY OF MALE REPRODUCTIVE ORGANS

Reproductive organs of male dog consist of testicles with epididymis and vas deferens, the prostate gland, the urethra and the penis. Testicle contains seminiferous tubules which produce spermatozoa, and the interstitial, which contains Leydig cells which are responsible for production of steroids, particularly testosterone, in sexually mature dog. Epididymis consists of single long duct in which spermatozoa undergo maturational process and become motile. The distal part of the epididymis, called *cauda*, serves as a storage for mature spermatozoa. Penis consists of pelvic part, called *glans penis*, which in dogs is approximately 5-15 cm long, depending on the size of the dog. Glans penis consists of two cavernous parts, *bulbus glandis* and *pars longa glandis*, which during the sexual arousal fills with blood, and creates an erection. Male dogs also have a penile bone, which is located dorsally of the urethra, which enables the coital intromission of non-erected penis (LINDE FORSBERG, 2001).

Testis is composed of two tissue types, the seminiferous tubules and interstitial tissue. Seminiferous tubules open into a collecting duct, called *vasa efferentia*, which then opens into the epididymis. Epididymis is divided into the head, body and tail. The tail of epididymis acts as a store for spermatozoa, which wait for ejaculation and leads into *vasa deferentia*, which also acts as a reservoir for spermatozoa. The vas deferens runs within the vaginal sac and its function is to transport the spermatozoa from testes to the penis (HEWITT, 1998).

The function of **accessory glands** it to add secretions to the spermatozoa, and it makes up a large volume the ejaculate. They may have a good effect on seminal plasma, such as correct pH. There are two types of accessory glands present in male dog, which are ampulla and prostate. Ampulla gland act as a reservoir of spermatozoa in *vasa deferentia* prior to opening into the urethra (HEWITT, 1998).

Prostate in dog contributes a large volume of watery secretion to the ejaculate, containing lactate, cholesterol and enzymes and very low concentrations of reducing sugars. Normally prostate is positioned near to the cranial rim of the pelvis and surrounds the terminal portion of ductus deferens, proximal part of urethra and neck of the bladder. It is symmetric and divided into 2 lobes by median septum. It is usually about 2 cm in diameter, but it varies with

the size and weight of the dog. Prostatic fluid is constantly secreted into excretory ducts which open into prostatic urethra (HEWITT, 1998).

Glans penis consists of two parts, which are differentiated by the location of erectile tissue. *Bulbus glandis* is composed of erectile tissue which surrounds the *os penis* and urethra, and the *pars long glandis*, has erectile tissue dorsally and longitudinally to the *os penis* and urethra. Dogs have an *os penis* present within a penis, which allows the male to achieve intromission before full erection. *Os penis* has a deep groove, in which urethra is situated (HEWITT, 1998).

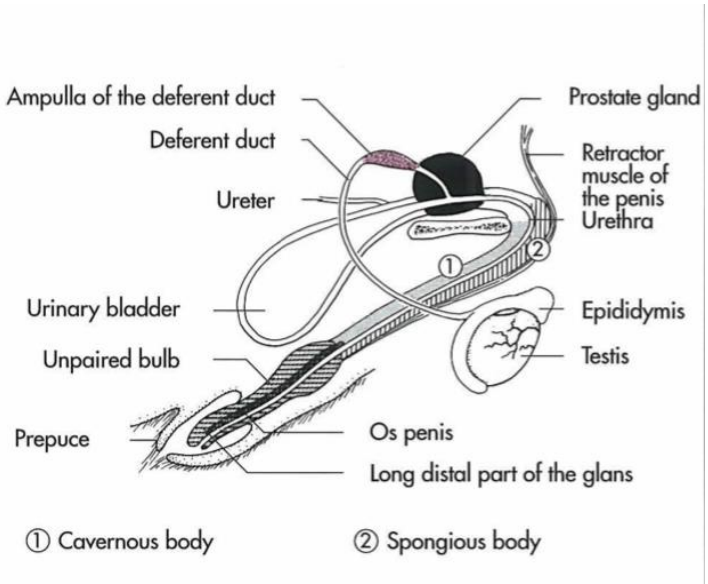

Figure 4. Genital organs of the male dog, schematic (KÖNIG and LIEBICH, 2004).

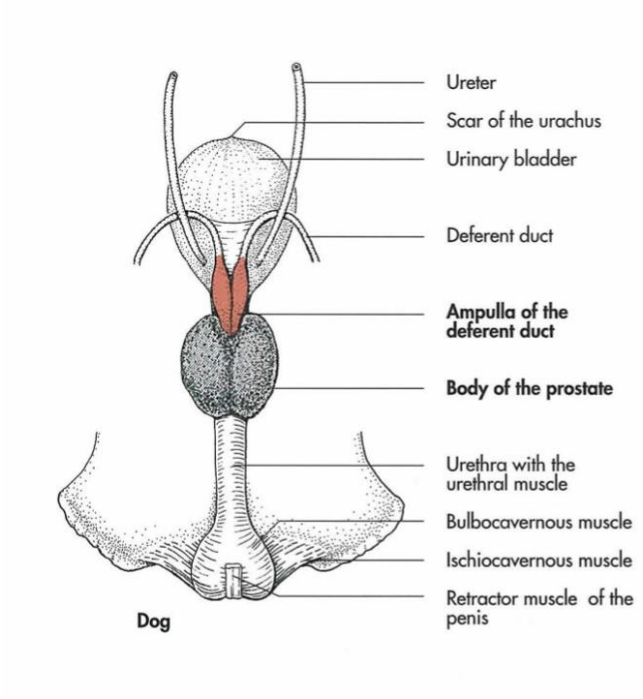

Figure 5. Accessory glands of the dog, schematic (KÖNIG and LIEBICH, 2004).

2.6 PUBERTY, MATURITY AND SENESCENCE IN MALE DOGS

Reaching a puberty in male dogs is not so obvious as in female dogs. It involves the display of a sexual behavior, the beginning of sperm production and ability to copulate. According to JOHNSTON et al. (2001), puberty in male dogs can be define as age at which a dog can successfully be induced to ejaculate. In male dogs, it usually occurs between 7 to 10 months of age. It is also dependent on the size of the dog, genetics, and other environmental factors (GOBELLO, 2014). The ejaculates from young dogs tend to contain higher percentages of abnormal spermatozoa (TAHA et al., 1981), therefore breeding the dog before reaching reproductive maturity is not advisable. On the other hand, senescence has an apparent effect on testicular function and spermatogenesis in dogs. With progression of senility, increased sperm defects, especially proximal droplets and lower percentage of normal spermatozoa in ejaculate can be detected which alter sperm mitochondrial function and sperm kinetics (RIJSSELAERE et al., 2007; ROTA et al., 2016; BRITO et al., 2020). This is related to failure in spermatogenesis or sperm maturation (CARREIRA et al., 2012). Spermatozoa from senile dogs are more sensible to cryoinjury, so cryopreservation should ideally be performed at reproductive maturity age (BRITO et al., 2020).

2.7 SEMEN COLLECTION AND EVALUATION

2.7.1 Semen collection

Semen collection should be performed in a quiet room and calm environment. For most dogs it can be done in the absence of a teaser bitch. However, collection can be done using female in estrus or using swabs or gauze sponges taken from vaginal secretion of estrus bitch. Teaser bitch is recommended because it increases the ease of collection and the volume of collection (PAYAN-CARREIRA et al., 2011). It is also recommended for the male dog to urinate before the semen collection.

Once the male dog is in the room, we should allow him to acclimate and interact with teaser bitch, if there is one. To avoid the distraction of the dog, there should not be to many people in the room, just owner or assistant together with the person who collects the semen. The person collecting the semen should stand on the side of the stud and allow male dog to sniff and lick the vulva and mount the bitch. Once the pelvic thrusting begins, the preputial sheet is gently massaged with gloved hand. In case the male shows no interest in the bitch and does not mount, massage of the dog prepuce at the level of *bulbus glandis* to stimulate an erection is performed. As partial erection occurs, the prepuce is quickly retracted to expose the penis. The goal is to make sure that the penis does not become erect within the prepuce as this is painful for dogs. After that penis must be encircled proximal to *bulbus glandis* and press must be kept. Semen is collected into pre-warmed plastic tube attached to a plastic sleeve or a funnel (LINDE FORSBERG, 2005). During pelvic trusting, rigid vials and funnels should be kept at distance to a penis, to avoid trauma. When this movements are finished and dogs start to lift its rear leg, penis should be rotated backwards for 180° and directed into the collection funnel (PAYAN-CARREIRA et al., 2011).

The dog ejaculates in 3 fractions: first the initial clear, slightly cloudy pre-ejaculatory fraction that contains few to no spermatozoa, second sperm-rich fraction (SRF) that comes from epididymis and testes, and third is clear, prostatic origin fraction and contains few to no spermatozoa. The third fraction is secreted in rhythmic pulses that can be palpable by the hand holding the penis and anal contractions are evident. The sperm-rich fraction should be thick, white and creamy liquid (PAYAN-CARREIRA et al., 2011). Once semen collection is complete, manual pressure to *bulbus glandis* is released. The male should not leave the room until penile detumescence is complete and the tip of the penis is not protruding. Detumescence can be hastened by allowing the male to lick the penis or walking the dog

away from the teaser bitch or area of collection (ROOT KUSTRITZ, 2010). Fractions should be collected separately. In most dogs, collections can be repeated within one hour, although the second sample is slightly diluted (PAYAN-CARREIRA et al., 2011).

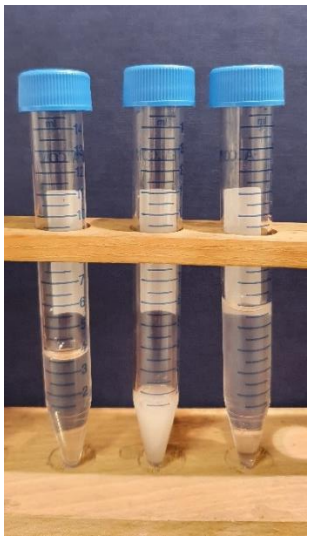

Figure 6. Fractions of dog ejaculate (courtesy of prof. Martina Lojkić)

Table 1. Main characteristics of dog semen fractions (PAYAN-CARREIRA et al., 2011).

Characteristics	1st fraction	2nd fraction	3rd fraction	Total ejaculate
Volume (mL)	0.5-5.0	1.0-4.0	1.0-80.0	2.5->80.0
Colour	Clear or opaque	Opalescent, greyish-white, white, milky-white	Clear, transparent	Opalescent
Consistency	Watery	Water- milky, milky	Watery	-
Character	Prostate secretion with admixture of epithelial cells urine, bacteria and sperm cells	Sperm cells suspended in seminal plasma	Prostate gland secretion	-
pH	6.3	6.1	7.2	6.3-6.7
Sperm concentration (10^6/mL)	-	300-2000	-	300-2000
Percentage of progressive motility	-	> 70%	-	> 70%

2.7.2 Semen evaluation

After collection and before preparation of semen for transport, semen should be evaluated macroscopically (volume, color, homogeneity, admixtures) and microscopically (motility, concentration, morphology and membrane integrity) (RIJSSELAERE et al., 2011).

Volume of first and third fraction can be variable, especially the third fraction that is controlled by the person who is collecting the sample. The volume of the semen is not an indicator for semen quality, but the volume measurement is part of calculation for total number of spermatozoa in the semen sample, which is the indicator for semen quality (ROOT KUSTRITZ, 2007). Volume is mainly dependent on the size of the dog and size of the prostate gland, the age, frequency of semen collection, the level of erotization and the volume of 3rd fraction collected. A decrease in volume is mainly observed in benign prostatic hyperplasia, prostatic cysts, some inflammatory lesions on prostate or testicles or inflammation of epididymis, vas deferens or urethra (PAYAN-CARREIRA et al., 2011).

Evaluation of **color** is subjective and dependent to the volume of third fraction (PAYAN-CARREIRA et al., 2011). Milky or cloudy is the normal appearance of sperm rich fraction, indicating that it probably contains spermatozoa, but should always be checked microscopically, because some dogs with azoospermia shed excessive numbers of fat droplets which can give appearance of normal semen. Clear sample usually contains no semen. Yellow color of semen indicates urine contamination. Brown semen discoloration can be indication of digested blood and light red discoloration is indication for fresh blood, which could be caused by prosaic disease or penile trauma (ROOT KUSTRITZ, 2007).

Values of **pH** can vary from 6.4 to 6.8. Seminal pH can change in case of presence of disease like prostatitis or if sample is contaminated with urine (ROOT KUSTRITZ, 2007).

Motility assessment is dependent on environmental temperature. Semen should not be exposed to quick temperature changes or contaminated equipment which can cause damage to spermatozoa (ROOT KUSTRITZ, 2007). The optimal temperature for assessment of sperm motility is 39°C (PAYAN-CARREIRA et al., 2011). Age of the dog may also influence the motility. Motility is evaluated under microscope. A drop of semen is placed onto a clean and warm glass slide and then covered with a coverslip. It is evaluated under 100-200x

magnification, and at least five fields should be evaluated. Percentage of progressive motility should be 70% or greater. Velocity can also be assessed, the normal motile spermatozoa should travel across the microscopic field of view in 2-3s. Nowadays, systems like computer-assisted sperm analyzers, also termed CASA, offer an accurate, rapid and simultaneous assessment of various semen parameters, including sperm concentration, total and progressive motility, slow, medium, rapid moving spermatozoa, linearity of sperm movement, the amplitude of lateral head displacement, beat cross frequency and various velocity parameters. These computerized measuring devices are good for detection of subtle changes in sperm motion, which cannot be identified by conventional semen analysis (RIJSSELAERE et al., 2012).

Morphology can be assessed using various techniques of slide preparation and staining, which can also affect morphology assessment (ROOT KUSTRITZ, 2007). Stains usually used include modified Giemsa stain (DiffQuik), Spermac stain, or eosin-nigrosin stain. Semen is smeared on a glass slide, air dried and stained and slide is then evaluated microscopically using oil immersion and objective x100 or x125. A minimum number of 200 spermatozoa should be counted and evaluated for presence of any abnormalities (PAYAN-CARREIRA et al., 2011). Age may also play a role in spermatozoa morphology. Morphologic abnormalities can be classified as primary and secondary or as major and minor. Primary occurs during spermatogenesis and secondary occurs during maturation of spermatozoa or sample preparation (ROOT KUSTRITZ, 2007). According to another classification in sperm abnormalities, major defect are defects that negatively correlate with fertility, and minor defects are unassociated with infertility (OETTLE, 1993).

Table 2. Main defects of the dog spermatozoa (PAYAN-CARREIRA et al., 2011).

	Primary spermatozoa defects	Secondary spermatozoa defects
Head	Macrocephalus, microcephalous, double, pointed, indented heads	Free, bent heads, swollen acrosomes, detaching acrosomes
Neck	Thickened, eccentric insertion	
Midpiece	Thickened, thinned, child, kinked, double midpiece	Bent midpiece, extraneous material surrounding midpiece, proximal, mid and vital cytoplasmic droplets
Tail	Thin, double, triple tail	Coiled, looped, kinked, folded, detached tail

Concentration of the semen is a parameter which is measured when performing semen evaluation in dogs but is not such a great indicator of semen quality. It is inversely connected with the volume of the semen collected. Concentration multiplied by volume is the total number of spermatozoa in the ejaculate. In dogs, normal total number of spermatozoa is higher than 300 million. The sperm concentration in normal canine ejaculate usually exceeds 80 $x10^6$ spz/mL. If the second fraction of ejaculate is collected separately, the sperm cells concentration in sperm-rich fraction varies usually between 200-600 x 10^6 spz/mL. It is generally assumed that the number of motile spermatozoa necessary for successful AI should be >150 $x10^6$ (LINDE FORSBERG, 1991). Total number of spermatozoa can decrease in case of frequent semen collection or with age, or sometimes even pain. CASA systems and optical density measurement are used for assessment of concentration of spermatozoa in dogs. Another traditional technique for assessment of spermatozoa concentration is hemacytometer, such as Thoma, Bürker or Neubauer chambers (ROOT KUSTRITZ, 2007).

Other cell types that can be found while assessing the semen quality can include some prostatic or urethral cells, immature germ cells, inflammatory or red blood cells. Even up to 2000 white blood cells/μL can be normal finding in first or second fraction (ROOT KUSTRITZ, 2007).

Another method that can be performed for semen evaluation is live-dead staining. This technique relies on a variable appearance of spermatozoa that takes up the stain and it is assumed that spermatozoa which take up the stain, have damaged plasma membranes and are marked as dead or nonfunctional. Stain that is commonly used is eosin-nigrosine stain. Interference with staining in case of presence of glycerol or fat globules or partial staining of spermatozoa can make inability in classifying those spermatozoa (ROOT KUSTRITZ, 2007). The normal dog semen consists of maximal percentage of 30% of dead sperm cells (PAYAN-CARREIRA et al., 2011).

Hypo-osmotic swelling (HOS) test is a method that involves submersion of spermatozoa into a hypo-osmotic medium, to identify spermatozoa with a functional and intact membrane. Spermatozoa that have intact plasma membranes will swell when fluid moves into the sperm cell, and this will cause the swelling and coiling of the tail. Hypo-osmolar solution includes sodium citrate, fructose, distilled water and sucrose solution. Incubation time can vary from 45-60 min but in some studies samples were evaluated after 1

min and did not differ in percentage of swollen spermatozoa from the ones evaluated after 60 min incubation (ROOT KUSTRITZ, 2007).

2.8 SEMEN PROCESSING AND PREPARATION

2.8.1 Extenders for chilled dog semen

After semen collection and evaluation, semen can be processed and prepared for transport using different semen extenders. Chilled semen is preserved at temperature of 4°C and should be diluted with proper extender (RIJSSELAERE et al., 2011). The goal of chilled semen storage is to preserve gametes at low temperature, without reaching the freezing point, which can cause harmful intracellular changes that affect the viability of the spermatozoa. The chilling preservation of semen is great alternative to semen cryopreservation (MARTÍNEZ-BARBITTA and RIVERA SALINAS, 2022). Spermatozoa are very sensitive to a rapid temperature fluctuation from 25 to 5°C, which can cause a cold shock and result in loss of selective permeability and integrity of the plasma membrane, release of intracellular enzymes and lipids, redistribution of ions and change in the membranes of the acrosome and mitochondria, loss of motility and diminished metabolism (ABOAGLA and TERADA, 2004). Metabolism of sperm is maximum at body temperature, and it decreases at room temperature (24-29°C). Metabolic waste products can increase acidity of semen causing permanent cell damage. For every 10°C decrease, cellular metabolism is reduced by 50 %, and at 5°C, there is only 10% of sperm metabolic activity left. Sperm cells with reduced metabolism have reduced lipid peroxidation and do not produce as many waste products which lengthen their lifespan. The use of semen extender during storage process is necessary to prevent damages, which could be caused by temperature decline, and to provide nutrient and energy to the cells (MARTÍNEZ-BARBITTA and RIVERA SALINAS, 2022). When semen is stored at 4-8°C, transition in the sperm plasma membrane from cooled crystalline to the gel phase is induced. To minimize this damage and to prevent cold shock, 10-20% egg yolk or skim milk is added to extender. Low density lipoproteins in egg yolk stabilize spermatozoa membrane, protecting the cell against cold shock. The other mechanism of preventing cold shock is slow cooling rate through temperature zone where transition from the crystalline to gel phase occurs. Besides protection from a cold shock, extenders must provide cells with energy substrates (sugars), buffer and electrolytes to maintain physiological pH and osmolality. To prevent growth of bacteria, antibiotics should also be added into semen extenders, mostly penicillin or

streptomycin. Activators can be added, to enhance sperm motility and to provide rapidly available sugars for sperm cells, and as well to provide a buffer against intense metabolic activity, which could cause decrease of spermatozoa longevity and intrinsic fertility (MARTÍNEZ-BARBITTA and RIVERA SALINAS, 2022).

Mammalian spermatozoa require exogenous substrates to preserve intracellular energy reserves, cell components and to support motility. They can obtain energy through mitochondrial oxidative phosphorylation and glycolysis by consumption of sugars, such as glucose, fructose, mannose and maltose. Glucose and fructose are two of the most used sugars for canine semen extenders (PONGLOWHAPAN et al., 2004). Sugars are included in semen diluents as exogenous energy substrates, as osmotic components and as cryoprotective agents. PONGLOWHAPAN et al. (2004) studied the importance of the source and concentration of the type of energy source molecule in sperm metabolism during refrigeration of canine semen and found that lactose is more efficient than glucose in obtaining energy levels higher in fresh semen. There are also indications that fructose may play a role as a sperm activator after ejaculation. YILDIZ et al. (2000), suggested that sugars allow maintenance of osmotic pressure and cryoprotective action. Based on this, it is expected that use of sugars in extenders could have positive effect on preservation of sperm progressive motility, maintenance of functionality and integrity of the acrosome and the sperm membrane (MARTÍNEZ-BARBITTA and RIVERA SALINAS, 2022).

Extenders can be purchased or home-made by veterinarian (RIJSSELAERE et al., 2011). Mostly used extender is Tris-fructose-citrate-egg yolk-based extender. There are also numerous commercial extenders available on the market such as CLONE Chilled Kit, Synbiotics (Zoetis), CaniPlus Chill 10, CaniPlus Chill LT and CaniPlus Chill ST (Minitube®, Germany).

2.8.2 Chilled Semen Preparation

Second, sperm-rich fraction is used to chill a sperm, which is then diluted with extenders in a proportion of 1:3 or 1:4 (1 mL of semen and 3-4mL of diluent). The sperm is usually centrifuged at 720 g for 5 minutes to remove seminal plasma (RIJSSELAERE et al., 2002). After that, sperm pellet is resuspended with 2-5 mL of pre-warmed (37°C) Tris-fructose-citrate extender with the addition of egg-yolk. Using larger volume for AI will lead to retrograde loss of spermatozoa. The semen is diluted depending on the total number of spermatozoa, and each dose should provide at least $200x10^6$ live, motile, morphologically

normal spermatozoa. Diluted semen is then placed in a beaker containing water (37°C) (Figure 7) and slowly cooled in the fridge to 4-5°C (RIJSSELAERE et al., 2011; LOJKIĆ et al., 2022). The diluted chilled semen can then be stored for up to 10 days at this temperature, depending on the type of extender and the semen quality (RIJSSELAERE et al., 2011).

Figure 7. A diluted dog semen placed in a beaker containing water (courtesy of prof. Martina Lojkić).

2.8.3 Shipment of Chilled dog semen

The chilled semen is packed in sterile 5 mL cryovials with screw cap which should be marked with dog's kennel club (KC) registered name, breed, microchip number and date of semen collection. In case the semen is to be used for 2 inseminations, sample should be split in two vials.

After preparation and chilling, the semen can be transported in a styrofoam box or Minitube neopore box (Figure 8). Extended sample is put into a box with ice packs, and the required temperature is maintained for up to 48 hours (RIJSSELAERE et al., 2011). Semen can be pre-chilled (4-5°C) in a fridge (LOJKIĆ et al., 2022) or can be placed directly into the transport box at room temperature adding two ice packs (-20°C) and cooled gradually within the box during transport (RIJSSELAERE et al., 2011). The transport box is non-returnable.

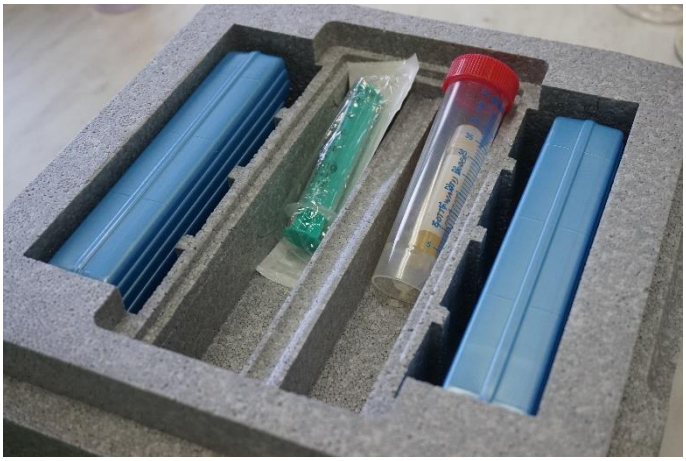

Figure 8. Transport box for chilled semen (courtesy of prof. Martina Lojkić).

2.9 FACTORS AFFECTING QUALITY OF CHILLED SEMEN

There are various factors which can affect the quality of chilled semen, for example age of the dog, semen collection and preparation, use of extenders and transit time. According to retrospective study of the relationship between canine age, semen quality, chilled semen transit time and season and whelping rate and litter size by LOJKIĆ et al. (2022), the following results were observed. In the study 43 dogs of 18 different breeds, 1.5-9 years old were included, and chilled semen transport over a 4-year period (2017 - 2021) was performed. All semen samples were collected manually without teaser bitch, evaluated and prepared with Tris-fructose-citrate extender (Cani Plus Chill, Minitube®, Germany) with addition of 20% (v/v) egg yolk and diluted depending on the total number of spermatozoa. Diluted semen was then cooled and packed. From total 43 transported chilled extended semen samples, 24 resulted in pregnancy and whelping (55.8%). The transit time of semen showed the effect on whelping rate but not on litter size. Eleven percent of bitches whelped when transit time was longer than 24 hours, in contrast to 67,6% of whelping rate when transit was completed within 24 hours. The effect of dog's age showed no effect on whelping rate or litter size. However, higher progressive motility was observed in dogs 3-6 years go are, compared to dogs <3 years of age. Percentage of morphologically normal spermatozoa was higher in dogs < 3years of age, compared to dogs > 6 years of age. The season of sperm transport did not show significant effect on whelping rate or litter size, although a lower whelping rate was observed in summer comparing to other seasons (LOJKIĆ et al., 2022)

There is another study about using three different transport containers for chilled semen and their effect on the quality of the semen, during different times of storage. The study showed that quality of chilled dog semen was dependent on the type of storage container, and the degree to which samples were affected was related to the period of storage. Containers used were Styrofoam box, a common Thermos flask and an Equitainer®. All three containers showed no differences in semen quality at 24h storage. At 48h storage, a higher progressive motility was observed in samples kept in Equitainer®, than in samples kept in Thermos. In samples kept in Equitainer® at 72h progressive motility was higher than in other two containers. Only samples kept in Equitainer® maintained similar levels of progressive motility between 24 and 72h of storage. Membrane integrity was independently affected by type of container and time of storage. Results indicated that use of Equitainer® is preferred when transport of chilled semen is expected to be longer than 48 hours (LOPES et al., 2009).

A study about the influence of glucose and fructose in the extender during long-term storage of chilled canine semen (PONGLOWHAPAN et al., 2004), revealed that the major effect of glucose and fructose in semen extenders for preparation of chilled canine semen, was to support sperm motility and movement patterns. In the study, the percentage of total and progressive motility remained high during the first 3 days and as expected, decreased with storage time. Preservation with higher content of sugars resulted in better maintenance of sperm motility and movement patterns, and addition of 70mM sugar to extenders showed beneficial effect on chilled semen. Fructose provided higher sperm motility over time, compared to glucose and the mixture at all concentrations, and appeared to be a preferable choice, by inducing higher percentages of total motility. Based on results from this study it is possible to use chilled sperm stored at 5°C for at least 8 days, when using egg yolk-tris extender with 70mM glucose or fructose (PONGLOWHAPAN et al., 2004).

2.10 ARTIFICIAL INSEMINATION

Artificial insemination is the method of depositing previously collected semen of stud dog into female reproductive tract so that fertilization can occur (PAYAN-CAREIRA et al., 2011; MASON, 2018). The advantage of AI is international exchange of semen and improvement of genetics, without the transport stress to the animals. AI also prevents the spread of sexually transmitted diseases, like *Brucella canis* and *Herpes* virus (LINDE FORSBERG, 2005; FARSTAD, 2010). It can be done using fresh, fresh chilled or frozen-thawed semen, and inseminated using different techniques which will be mentioned later. Using chilled semen is

excellent way to accomplish international breeding, without cost and stress related to dog transport. The success of AI is dependent on proper selection of male and female dogs, proper preparation of semen, semen quality, accurate ovulation timing, proper insemination timing and technique and good communication between veterinarian and a client.

2.11 OPTIMAL TIME FOR ARTIFIFCAL INSEMINATION

Proper timing of ovulation, and subsequently insemination, is crucial for obtaining successful pregnancies and adequate number of offspring per litter. Due to specific physiology, it is very important to detect the ovulation, in order to decide the optimal insemination time, depending on the type of semen used. There are different test and methods which can be used for detecting fertile period of the bitch, and therefore the optimal breeding time. These include clinical signs, vaginal examinations including vaginoscopy and cytology, measuring LH and progesterone blood levels and, in some cases, ultrasound examination. None of these methods detects ovulation directly, but by their combination it is usually easy to detect beginning and end of ovulatory period and set the optimal time for insemination.

Clinical signs indicating estrus and fertile period include vulvar swelling and vaginal discharge, and behavior of the bitch in presence of male dog. During the optimal time bitch is receptive to mating and allows male dog to mount her. Bitch shows typical sexual reflexes like lateral curvature of the rear quarters positioned towards the male, arching of the tail to the side and vertical movement of the genital orifice (HART, 1970; BEACH et al., 1982). The increased turgidity of the vulva that is present in proestrus reduces and the vulva becomes softer. Hemorrhage diminishes and discharge is lighter in color (LINDE FORSBERG, 1991).

Vaginal cytology is a quick, simple, and inexpensive diagnostic method to determine optimal time for insemination and can be useful in combination with other methods. Cellular changes which can be found are caused by changes in plasma estrogen concentration, so ovulation cannot be detected (LINDE FORSBERG, 1991). However, method is excellent for monitoring and determining the stage of estrus cycle. Vaginal samples are collected using moistened cotton swab and then rolled onto a microscopic slide, stained, most commonly with Diff-Quick method, and evaluated under the microscope (MASON, 2018). Preferred place of

taking the swab is cranial vagina. On vaginal smear 4 types of cells can be noticed: parabasal, intermediate, superficial and anuclear cell types.

During proestrus, increased cornification can be seen on vaginal smear, due to increasing levels of estrogen secreted from follicles, which results in hyperplasia of the vagina. Due to vaginal hyperplasia, surface cells of vaginal wall are moved further from the blood supply of the submucosa, which results in reduced oxygenation and consequent death of these cells. This process is visible by changes in cells such as enlargement of cells, change in their cytoplasm:nucleus ratio, nuclear pyknosis and finally cornification of the surface cells. With the start of estrus, the percentage of nuclear cells increases to 50% of the cornified cells, while during fertile period they account for more than 90 % of all cells in smear. Approximately 6 days after ovulation the influx of neutrophils and parabasal cells is seen, with abrupt reduction in the percentage of cornified vaginal epithelial cells, and this indicates the end of the fertile period (estrus) (JEFFCOATE and LINDSAY, 1989; MASON, 2018).

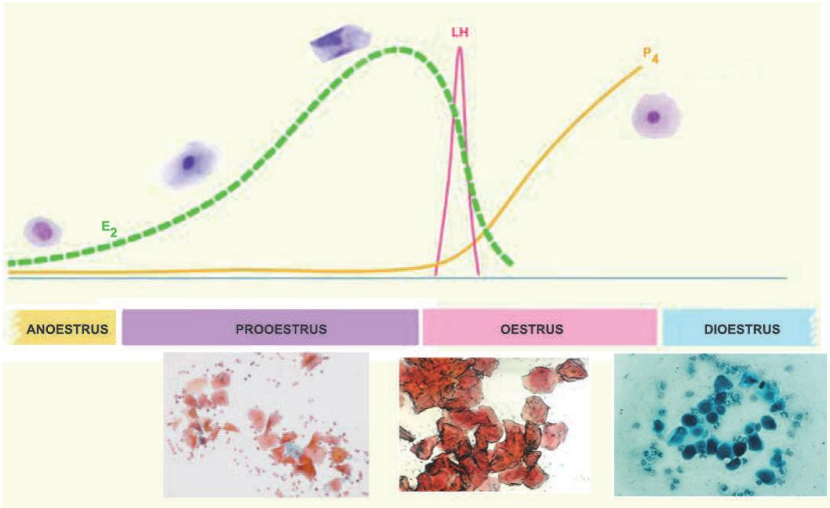

Figure 9. Type of cells according to the phase of the cycle (PAYAN-CARREIRA et al., 2011).

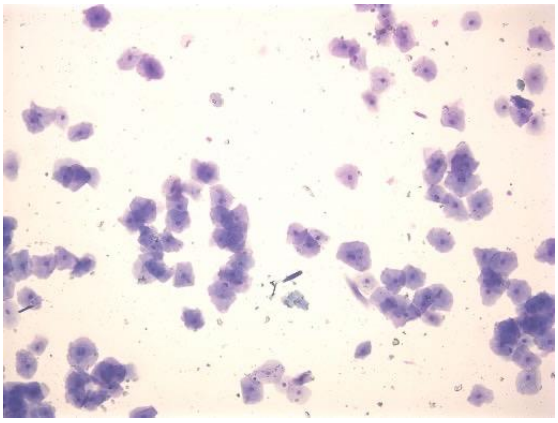

Figure 10. Mid to late proestrous canine vaginal cytology (Wright's stain, 400×), (ROOT KUSTRITZ, 2020).

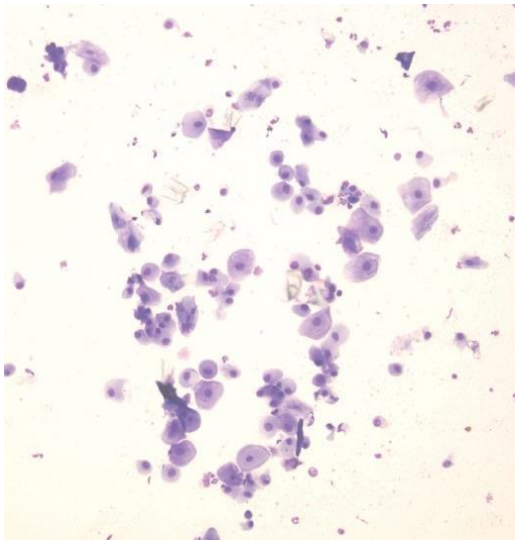

Figure 11. Early proestrous canine vaginal cytology (Wright's stain, 400x), (ROOT KUSTRITZ, 2020).

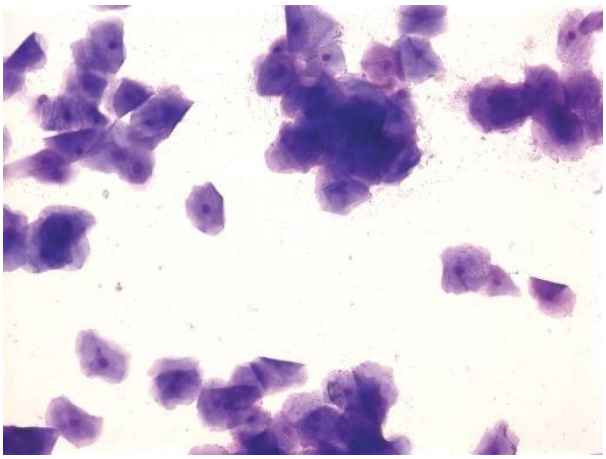

Figure 12. Estrous vaginal canine cytology (Wright's stain, 400×), (ROOT KUSTRITZ, 2020).

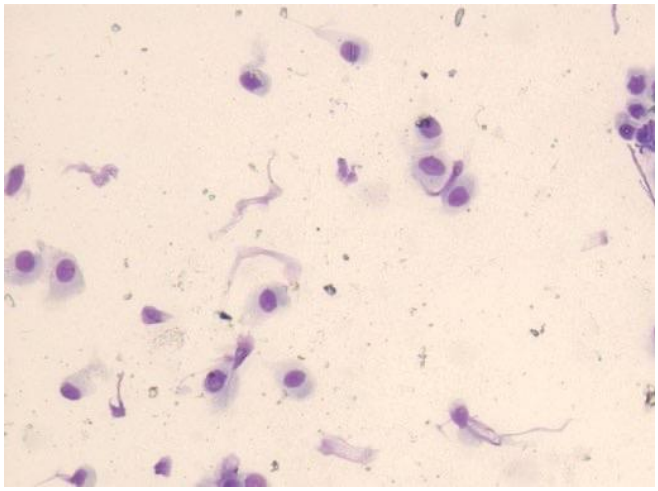

Figure 13. Anestrus vaginal canine cytology (Wright's stain, 400x), (ROOT KUSTRITZ, 2020).

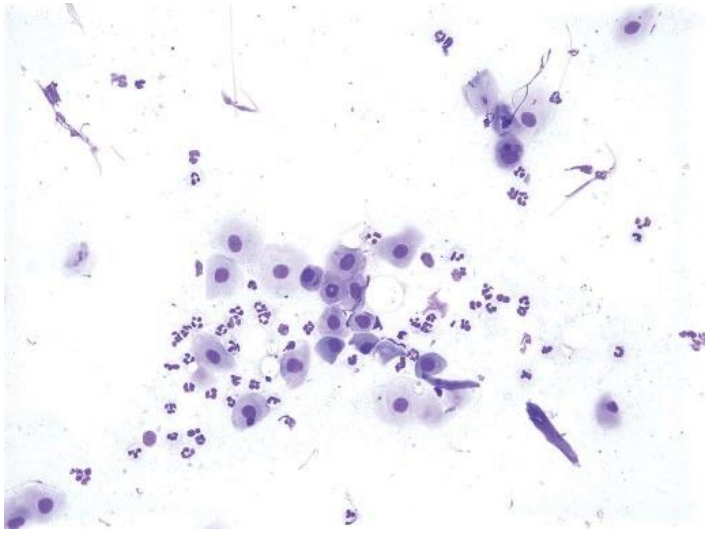

Figure 14. Diestrous vaginal canine cytology (Wright's stain, 400x), (ROOT KUSTRITZ, 2020).

LH measurements can be performed, by qualitative or quantitative assays. Level of LH remain undetectable until the LH surge, and because the length of LH surge can vary in bitches from 24-60 hours, daily serum analysis is required. There is an increasing phase that last up to 12-24 hours, followed with decline over 12-36 hours (CONCANNON, 2011). Because the levels of LH can vary from 4-14 ng/mL, and duration is relatively short, even daily testing can miss the LH surge. On average, LH surge occurs 5 days before the oocyte being ready to be fertilized (MASON, 2018).

Progesterone assay is a useful method in detecting optimal breeding time. The level of progesterone increases from the LH surge and can be used as an indirect indicator of ovulation. Progesterone rises as a result of preovulatory granulosa cells luteinization, and corpora lutea development after ovulation. Progesterone is commonly measured from serum. Progesterone level of around 6 nmol/L (2 ng/mL) generally describes the occurrence of LH surge, and ovulation at 15-25 nmol/L (4-8 ng/mL), which is complete when the values are greater than 30 mol/L (10ng/mL). After ovulation, progesterone continues to increase, and influences the closure of the cervix, which is believed to occur at progesterone levels of 60-90 mol/L (20-30ng/mL), but there are different values described. The closure of cervix helps protect the uterus from infection and provides an ideal environment for fertilization. If insemination is to be done after cervical closure, the intrauterine insemination should be performed. Progesterone is commonly measured by immunochemiluminiscence or enzyme-linked immunoassay, with results being obtainable within 30-45 minutes. The blood is sampled every second or third day. The increase of sampling interval will decrease the accuracy of ovulation detection (FARSTAD, 2010). However, described changes of the values can be different in each bitch, and individuality of animal must be taken into consideration when determining the optimal breeding time (MASON, 2018).

Vaginal endoscopy can be used to determine the fertile period, but it does not determine the accurate timing of ovulation. This method also requires expensive equipment and knowledge to perform, and sometimes sedation is necessary. On the other hand, it can be useful in detecting some anatomical abnormalities which could influence the normal reproductive performance (PAYAN-CARREIRA et al., 2011). Some specific morphologic changes on the vaginal mucosa can be seen, due to fluctuation of estrogen and progesterone concentrations, and these changes could also be assessed for determination of optimal breeding time (LINDSAY, 1983; JEFFCOATE and LINDSAY, 1989; GOODMAN, 2001).

The most distinct finding is crenulation of mucosa. Smooth mucosa becomes wrinkled and shrunken, vaginal folds become smaller, creating flattened, secondary folds and specific cobblestone appearance of vagina, or crenulation (MASON, 2018). There is loss of fluid from vaginal mucosa and submucosa, and as result vagina appears wider compared to proestrus. Maximal intensity of shrinkage of vaginal mucosa is seen between 3-8 days of estrus cycle. This finding responds to maximal number of anuclear cells in vaginal smear and is indicative to fertile period. In diestrus vaginal folds appear flat and round (PAYAN-CARREIRA et al., 2011). These changes could also be assessed for determination of optimal breeding time (LINDSAY, 1983; JEFFCOATE and LINDSAY,1989; GOODMAN, 2001).

Ultrasound examination is not routinely used as a method for optimal breeding time determination, because of difficulty in accessing ovaries by ultrasound in bitch. Several changes are described, but they are not specific enough to have diagnostic value in determination of ovulation (FONTBONNE and MALANDAIN, 2006), since ovarian follicles do not differ much in the immediate pre- and post-ovulatory period.

After ovulation, ova require 96-108 hours to finish maturation and gain fertilizing ability (TSUTSUI, 1989; CONCANNON, 2011). Fully functional ova, or secondary oocyte, has a life span of 24 to 48 h (TSUTSUI, 1989). Therefore, timing of insemination differs with different type of semen used. The more processed semen sample, the closer timing to fertile ova must be. To determine optimal breeding time, it is recommended to use combination of techniques mentioned above. When using fresh semen, insemination should be performed on the day of ovulation, and second insemination 2 days later, if this is possible. However, timing of artificial inseminations can vary between person who's performing it, according to their experience, technique of inseminations and number of inseminations, so regimes for canine AI can vary with authors (ROOT KUSTRITZ, 2003). Days 4-7 after LH surge represent the fertile period, with peak fertility on days 5 and 6. According to PAYAN-CARREIRA et al. (2011), insemination with chilled semen is performed once or twice, 2-4 days post ovulation at the progesterone level of around 8-15 ng/mL, with expected 24-72 hour survival of semen. The expected fertility is 80-90%, either with transcervical or vaginal deposition. When using chilled semen, the quality of the semen and the site of deposition are very important factors for the success of AI (PAYAN-CARREIRA et al., 2011).

2. 12 INSEMINATION TECNIQUES

There are different techniques available for AI, depending on the type of semen used and timing: deep vaginal insemination and intrauterine insemination, either via non- surgical transcervical insemination or surgical insemination, by laparotomy or laparoscopy (PAYAN-CARREIRA, 2011). Some authors describe intratubal semen deposition as one of surgical techniques (TSUTSUI et al., 2003): With chilled semen, deep intravaginal and transcervical insemination can be used, while surgical methods are used mainly with frozen-thawed semen.

Deep vaginal insemination is one of the mostly used methods for insemination, mainly with fresh semen. It is performed with plastic catheter or a commercial catheter in flexible latex tube presenting an inflatable balloon at the tip, which prevents a semen back flow when inflated (LINDE FORSBERG, 2005; FARSTADT, 2010). Before AI, perineal and peri-vulvar area must be cleaned. Bitch is placed in standing position. The insemination catheter is carefully introduced into the vagina, steeply upward at the beginning until pelvic brim is passed, and then at the horizontal angle carefully pushed forward (FARSTAD, 2010). Care must be taken not to catheterize the urethra. For that reason, gloved finger can be inserted into vagina and secure the catheter while it is moved cranially through the cranial portion of vagina, which is bordered by the dorsal medial folds. Once catheter is in the paracervical area close to the external cervical os, semen is slowly deposited (PAYAN-CARREIRA, 2011). During AI, the bitch is held with hindquarters up and head down in the angle 45-60°, to ensure that the semen will not be expelled with the backflow. It is also recommended for bitch to stay in this position for 5-20 minutes after AI (PAYAN-CARREIRA, 2011). PINTO et al. (1998) have found that reducing hindquarter elevation time has little effect in pregnancy rate and litter size. Greater success in pregnancy is established when vaginal massage with finger guiding urethral opening is performed, thus causing vaginal contractions, and mimicking natural breeding conditions.

Different devices can be used for performing vaginal insemination in bitches:

AI pipette for dogs, a rigid plastic pipette with no cuff. Rigidity and small diameter of catheter enable its passage to the level of cervical os. As there is no cuff, no vaginal distension or prevention of semen backflow is possible. Comes in different lengths.

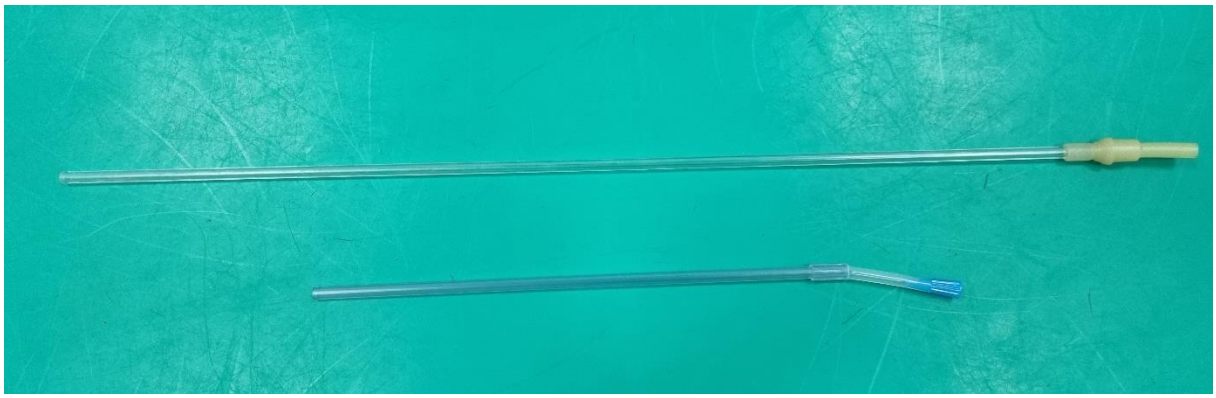

Figure 15. AI pipette for dogs (courtesy of prof. Martina Lojkić)

Osiris catheter, which is a rigid catheter with inflatable cuff at the distal opening, usually around 25 cm long and 5 mm in diameter. Once the catheter is positioned correctly, the cuff is blown up though the external valve, which will cause vaginal distention and prevention of semen backflow around the catheter. Semen is deposited through the lumen and syringe should be left attached, again to prevent back flow of semen. This type of catheter is goof for small and medium sized dog breeds, but for larger dog breeds catheter is too short and the cuff is too small (MASON, 2018).

Figure 16. Osiris catheter for AI (MASON, 2018).

Foley catheters are flexible with inflatable cuff near distal opening and come in different sizes and is adaptable to different sizes of dogs. Semen is introduced into the vagina in the same manner as with Osiris catheter, but due to its flexibility, it is difficult to position distal opening of the catheter close to the cervix (MASON, 2018).

Mavic catheter is plastic catheter with stylet through the center. It has inflatable cuff at the distal opening, and the stylet, through which semen is inseminated, contains one-way valve,

which prevents the backflow of semen. Inflatable cuff allows vaginal stimulation and prevents back flow of semen. These catheters are available in 3 different sizes, suitable for all sizes of bitches (MASON, 2018).

Figure 17. A 250-mm Mavic catheter for vaginal insemination in the bitch (MASON, 2018).

Transcervical insemination, is the most widely used technique for AI with chilled and frozen-thawed semen. There are two different techniques described: endoscopic- assisted transcervical and Norwegian method. In both procedures, insemination can be performed more than once, resulting in increased offspring per litter (MASON, 2018).

Endoscopic-assisted transcervical insemination EIU, or also known as transcervical insemination (TCI). In the first days, cystoscope was used, nowadays, most operators use the uterorenoscope, because it is longer and thinner than the cystoscope, and can be used in all size of bitches. This procedure is performed in bitch standing and restrained on the table. Apart from uterorenoscope, additional light source and a camera which projects the image onto a monitor are needed. Endoscope is passed into the vagina, and air is introduced in the vagina. Vaginal shunt can be used to maintain vaginal distension. The endoscope is slowly moved cranially past the dorsomedian folds to the cervix. The cervical os is commonly visualized on the ventral part on the cervix. Once the catheter is in the cervix, the stylet is removed, and catheter is introduced into the body of the uterus. Then the semen is slowly and carefully inseminated thought the catheter, taking care that no leakage occurs at the cervical os, and massage the vulva to stimulate the uterine contractions (MAKLOSKI, 2012). This method secures optimal place for semen deposition and results in very good pregnancy rates, it is simple to learn but each step of the process must be closely monitored (MASON, 2018).

Norwegian (or Scandinavian) method, using Norwegian catheter- another type of catheter also used for transcervical/intrauterine insemination. It consists of outer nylon sheet, with inner metal stylet, and has blunted and rounded distal tip. This procedure can be used in any size bitch, without anesthesia. Bitch is restrained on the table in standing position. The operator with one hand transabdominally palpates the cervix, and with other hand passes the catheter into the vagina, and while holding the cervix, the stylet is passed into the cervical os and thought the cervix. Once the catheter is placed in the correct position, the semen is inseminated through the stylet into the uterine lumen. This procedure is quick and simple, and inexpensive, and can be used for fresh, chilled or frozen semen, but most published reports are for frozen semen (MAKLOSKI, 2012; MASON, 2018). Disadvantage is steep learning curve, and the process is difficult in large breeds (MASON, 2018).

Surgical insemination can include conventional, laparotomic intrauterine insemination or laparoscopic insemination (MAKLOSKI, 2012). These procedures have some welfare issues, so they are illegal in some countries (Norway, Sweden) (MASON, 2018). On the other hand, when female's cycle is properly managed, success rates of both methods can reach 100 %, including bitches with known reproductive issues (BRITTAIN et al., 1995, SILVA et al., 1995).

Conventional laparotomy, commonly also known as surgical AI or surgical implant (SIU), is the insemination of semen into the uterus. This procedure is performed under general anesthesia by laparotomy. Bitch is positioned in dorsal recumbency, and midline incision is made of 25 mm caudal to the umbilicus, toward the caudal direction. The uterus is exteriorized and inspected. For insemination, 22-gauge IV catheters can be used. After placement of the catheter, the uterus is occluded above the cervix, and the syringe with the semen is attached onto catheter and inseminated slowly into the uterus. After insemination, the uterus is massaged to stimulate uterine motility, which is decreased due to anesthesia. The catheter is removed, and then the abdomen is closed routinely (MASON, 2018).

Laparoscopic insemination is not a common procedure in clinical practice, due to high costs, and knowledge and experience needed, and due to lack of advantages of this procedure over SIU (MASON, 2018). The semen is introduced into the uterine horn through 18–22-gauge catheter under the visualization of laparoscope, connected to an electronic light source and camera, inserted into abdominal cavity. The uterine horns are visualized and elevated close to the abdominal wall so catheter can be inserted. The procedure also requires general

anesthesia. In comparison with the SIU, laparoscopic insemination is less traumatizing for the bitch thus allowing faster recovery. It also provides better protection against surgical risk of infection, as abdominal cavity is not opened (SILVA et al., 1995).

Intratubal insemination involves deposition of semen into the oviduct; it is as invasive as other laparotomies for surgical insemination, allows smaller sperm number to be inseminated but results in lower pregnancy rates compared to surgical or transcervical insemination (TSUTSUI et al., 2003).

2.13 SUCCES OF ARTIFICIAL INSEMINATION USING CHILLED SEMEN

To obtain desirable and good success rates for artificial insemination with chilled semen it is important to estimate proper timing of insemination, the use of adequate number of viable sperm cell per dose, good semen preparation and handling and adequate deposition of semen in the female reproductive tract. It is important to use combinations of tests to estimate optimal breeding time. Depending on authors, and on insemination technique, the success rate of AI with fresh chilled semen in dogs is around 80-90% when transcervical insemination is used, and around 65 -90 % when deep vaginal insemination is used (MAKLOSKI, 2012).

A study of LINDE FORSBERG and FORSBERG (1989) on results of 470 artificial inseminations showed that pregnancy rate was higher in bitches receiving chilled extended (83.3%) semen compared to frozen (69.3%) semen. Litter size was 23.3% smaller in bitches receiving frozen semen.

According to PINTO et al. (1999) pregnancy rate for bitches inseminated with chilled extended semen was not statistically lower from pregnancy rate of bitches inseminated with fresh semen. Pregnancy rate was 94% and 95% for chilled and fresh semen, respectively.

Whelping rates from 527 artificial inseminations using chilled extended semen were reported to be 45.7% when the semen was deposited in the cranial vagina and 65.6% by transcervical intrauterine semen deposition. The average litter size was 5.8 ±3.0 pups by vaginal AI and 6.4 ±3.2 by intrauterine AI (LINDE FORSBERG and FORSBERG, 1993). The results of this study emphasize the importance of intrauterine semen deposition when using chilled extended semen. The study on 2210 AIs in dogs by LINDE FORSBERG (2002) showed similar whelping rate and litter size for fresh and chilled semen (48,9% and 49% of whelping rate and 6.5 and 6.4 litter size, for fresh and chilled semen respectively). In contrast to that, whelping rate for frozen semen was higher (53.8%). Reason for this is that more

precise timing of insemination and intrauterine deposition of semen are applied when using frozen semen. Whelping rate of 55.8% and 4.71 pups per litter are reported by LOJKIĆ et al. (2022).

2.14 LEGISLATION AND ETHICAL ASPECTS ON USE OF CANINE ARTIFICIAL INSEMINATION

Demand for canine AI is increasing world-wide, as for medical and breeding management reasons, like improvement of genetics, reduced risk of infectious disease, travel distance or failure of natural mating, which can occur due to some anatomical defects or behavioural reasons. There is still risk of transmission of some diseases with AI, which include viral canine herpesvirus and rabies, bacterial leptospirosis and brucellosis and parasitic, leishmaniasis and toxoplasmosis (QUARTUCCIO et al., 2020). There is also a risk of transmission of genetic disease by AI, so any dog used for AI should also be screened for inherited diseases if a DNA-test for the disease/functional disability is available. The breeding stock should be tested to avoid mating of two carriers. Screening should be recommended for diseases and breed, where disease has a major impact on dog's health. Dog used for breeding must fulfil all breed demands and standards, like genetic testing and radiographic screening. Dogs with congenital and genetic faults, like congenital deafness, cleft palate, substantial dental or jaw anomalies, eyes disease, heart disease, cryptorchidism, hip and elbow dysplasia, and other, should not be reproduced.

When it comes to the national regulations and legislations, different countries have different requirements. There are several countries in the world which have no legislation or requirements regarding AI and the use of chilled or frozen-thawed semen. Most kennel clubs follow the regulations from FCI (International Canine Federation) who recommends that AI should only be done in healthy dogs with proven fertility. FCI regulations also limit the use of dogs presenting disease, which could be transmitted to the following generations, and cause some eliminatory defects regard to the breed standard (QUARTUCCIO et al., 2020). On the other hand, some countries like Australia and New Zeeland have strict regulation related to importing canine semen, and if semen does not meet all requirements will be refuse entry and either returned to the country of origin or ordered to be destroyed. Some countries require an import permit, while others require only veterinary health certificate. Both national legislation and kennel club regulations may change at any time, so it is advisable to check the latest

regulations and rules at importing country before planning the semen collection and shipment, so that necessary blood tests and certificates can be made in accordance with requirements.
For EU, the shipment of canine semen must be accompanied with official veterinary certificate. The official certificate is issued by veterinarian, and contains information regarding species, breed, date of birth, sex, colour and identification number (microchip number or ID-tattoo marking) of the donor dog, and also a statement that the dog has normal testicular status. Certificate also includes a semen assessment form with instructions and recommendations of how to prepare/use the semen. Microchip number of the donor must be written on all records, laboratory reports certificates and semen containers. The official, KC registered name of the donor dog should be used in all documents. Quantity of semen should be reported as well, contact of the imported and exporter, and date of collection. Donor dog must be examined prior to semen collection and should not have a history of infectious or contagious disease in the last 30-45 days. Movement of dog semen between EU Member States and to EU from third countries is under Regulations (EU) 2021/1880, (EU) 2020/686 and (EU) 2016/429 and require TRACES-NT certificate signed by Official Veterinarian (ANONYMUS, 2021).

Ethical aspects of AI may be positive regarding the impact on disease control, but there are some concerns regarding surgical procedures. When choosing the type of inseminations, welfare of bitch, has to be respected firstly, and secondly considering benefits and risk of surgical procedure. Surgical AI, it is banned in some EU countries (Norway, Sweden) and UK, due to welfare laws. Inbreeding is also an important ethical concern of the use of AI (QUARTUCCIO et al., 2020).

3. CONCLUSIONS

1. AI with chilled semen requires careful planning which include determination of optimal breeding time and shipment of semen on time.

2. To determine optimal breeding time, it is recommended to use combination of tests, like observing the clinical signs, vaginal cytology, and progesterone assay.

3. Different types of extenders, transport containers and transit time can affect semen quality and fertilization ability. Adequate number of viable sperm per dose provides success of AI.

4. Intrauterine deposition of semen results in higher pregnancy rate and litter size making endoscopic-assisted intrauterine insemination preferable method of insemination.

5. Ethical consideration and welfare of the bitch must be considered during artificial insemination. Practitioners should acquire knowledge on reproductive physiology of male and female and collect the semen and inseminate the female without risking animal welfare.

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5.SUMMARY

Zabret, A. (2022): Artificial insemination with chilled semen in dogs

Artificial insemination is a useful method to improve genetic traits in different dog breeds. The use of fresh, chilled semen has become very popular among dog breeders. Chilled semen is especially suitable for short term storage and short distance transportation, i.e., 24-48 hours. This method allows for reducing travel-related stress and inbreeding, by using genetically superior dog semen all over the world. It is also a good way to prevent the spread of sexually transmitted disease. By mixing semen with extender and cooling to 4°C, fertility remains preserved for a long time. Semen extenders protect the spermatozoa from cold shock, provide energy substrates and maintain constant pH and osmolarity. Mostly used extender is Tris-fructose-citrate-egg yolk-based extender providing sperm longevity for 3-10 days. AI with fresh chilled semen can be performed using different techniques, such as deep vaginal insemination, or preferably by intrauterine insemination, which can be performed by using non-surgical transcervical catheterization or by surgical semen deposition by laparotomy or laparoscopy. Vaginal insemination provides adequate pregnancy rate, but intrauterine insemination gives higher pregnancy rate and litter size. To perform AI successfully, anatomy of female reproductive tract and physiology of estrus cycle of the bitch must be known so that procedure could be performed at appropriate time and by using the most appropriate insemination technique and place of semen deposition, according to the individual case.

Key words: dogs, chilled semen, extenders, optimal breeding time, insemination technique

6. SAŽETAK

Zabret, A. (2022): Umjetno osjemenjivanje rashlađenim sjemenom u pasa

Umjetno osjemenjivanje (UO) omogućuje poboljšanje genetskih osobina kod različitih pasmina pasa. Korištenje svježeg ohlađenog sjemena postalo je popularno među uzgajivačima radi brojnih razloga. Pogodno je za kratkotrajno skladištenje i transport sjemena na kratke udaljenosti, unutar 24-48 sati. Smanjuje parenje u srodstvu i stres uzrokovan putovanjem životinje na parenje, korištenjem sperme genetski vrijednih mužjaka iz svih krajeva svijeta. Isto tako, sprječava širenje spolno prenosivih bolesti. Ohlađeno sjeme čuva se na temperaturi od 4°C, gdje ovisno o vrsti razrjeđivača ostaje sačuvano kroz duže vremensko razdoblje. Razrjeđivači štite sjeme od temperaturnog šoka, osiguravaju energiju te održavaju stabilan pH i osmolarnost. Najčešće se koristi Tris-fruktoza-citratni razrjeđivač s dodatkom žumanjka koji omogućuje preživljavanje spermija kroz 3 do 10 dana. UO s ohlađenim sjemenom može se izvesti različitim tehnikama kao što su duboko vaginalno osjemenjivanje ili pak intrauterino osjemenjivanje koje se može izvesti transcervikalnim (TCI) ili kirurškim polaganjem sjemena laparotomski ili laparoskopski. Iako se vaginalnim osjemenjivanjem postižu dobri rezultati, TCI predstavlja metodu izbora radi veće stope skotnosti i veličine legla. Za uspješno izvođenje UO potrebno je dobro poznavati anatomiju ženskog spolnog sustava i fiziologiju spolnog ciklusa kuje, kako bi se odredilo optimalno vrijeme za osjemenjivanje i primijenila najprikladnije tehnika osjemenjivanja, ovisno o pojedinačnom slučaju.

Ključne riječi: pas, ohlađeno sjeme, razrjeđivači, optimalno vrijeme za parenje, tehnike osjemenjivanja

7. BIOGRAPHY

Ajda Zabret was born on 1st of December1997, in Ljubljana, Slovenia. She finished Veterinary Technician high school in 2016, and enrolled Veterinary Faculty in Zagreb in the same year, as one of the students of the first generation of international program.