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**AI of horse with cooled shipped and frozen semen.
Practical experiences.**

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1 Introduction:

1.1 General considerations of artificial insemination in horse breeding

I have investigated the use of artificial insemination in horses, comparing the use of cooled shipped and frozen semen. AI in horses has become very popular the last few years. I have investigated the different methods, comparing the benefits and drawbacks of it, focusing on the use of cooled shipped and frozen semen in horses. For 9 months I enjoyed staying at the Doubletree Horse Farm in Colorado, where they breed Quarter Horses using both fresh, cooled and frozen semen. I got to perform much practical work, including collection of their three stallions, extending and storing the semen, and inseminating the mare at the farm.

1.2 The facts about breeding soundness examination and the timing and procedure of insemination

Several factors are essential to consider before you start using both the stallion and the mare for AI. The stallion needs a thorough examination for the breeding soundness as well as confirmation showing that he has sufficient semen quality. He must be checked to make sure that he does not carry any diseases that can be venereally transmitted to the mare, or any other lesions that may reduce the longevity of the stallion as a sire. The semen sample must have appropriate cooling and storage after collection. The libido and mating ability of the stallion, and any congenital defects that may transmit to the offspring should be detected.

1.2.1 Mare management

The mare also needs a satisfactory soundness examination, showing that she is fit to carry out a healthy pregnancy. Induction of ovulation and an accurate prediction of ovulation are both extremely important to carry out. This way the timing of insemination will be as correct as possible in correlation to ovulation. Before insemination, it is extremely important with appropriate storage, thawing and handling of semen, as well as a good insemination technique. After insemination, it is relevant to examine the mare being bred and treat her if required. In order for everything to work successfully, we need to be well prepared to set everything up for success. I have included the use of artificial light programs for a predictable and correct timing of insemination. The basics of the mare estrous cycle is essential to understand from a practical view in order to be able to correctly time the ovulation and manipulate by using drugs if necessary. We should take benefit of the knowledge that exist on these topics and use it as a tool for completing a successful breeding program.

1.2.1.1 Artificial lights

Artificial lights can be used to hasten the onset of the breeding season. It should be started 2-3 months prior to the desired first breeding date. It should be noted that artificial lights can terminate the winter anestrus, but it cannot affect the spring transition period. There are two light programs that are commonly used. One of them includes a total 16 hours of continuous light, which can be both daylight and artificial light. It can be added at the end and/or the start of the day. The other program turns lights on for 1 hour about 9.5 hours after sunset. Shedding of hair coat can be expected within 30-60 days, and the ovarian activity leading to ovulation happens within 60-90 days after starting the light program. The interval from starting the lights to the first ovulation is often longer for thin mares with inactive follicles, and shorter for fatter mares with more active follicles. The temperature is not a factor, so the artificial lights can be used outdoors. It is advisable to use an automatic timer, as any interruption in the program will have a negative effect and possibly set the mare back in time. Because periods of short days are required artificial lights cannot be used all year. (KUBIAK, 2013)

1.2.2 The estrous cycle of the mare and practical aspects of inducing ovulation

The average estrus of the mare is 5 days, but it is important to know that this varies a lot between each mare. The diestrus period is on average 16 days, and the whole cycle is 21 days. The foal heat usually takes place 9 days after foaling, and it lasts for an average of 4 days. During estrus the follicle grows about 3-5 mm per day, and it has a size of about 35-55 mm at ovulation. "The mare ovulates approximately 24 hours prior to the end of estrus."(MACPHERSON, 2010). Prediction of ovulation is not always a simple task due to the variations from horse to horse, and it is often useful for the veterinarian to have some knowledge about the previous pre-ovulatory diameter as these can differ remarkably between the mares. Some important tools to make the prediction as accurate as possible are daily rectal and vaginal palpation, and the use of ultrasound. One of the hardest parts of AI is to determine the optimal time to inseminate the mare. Therefore, hormones are often used to induce ovulation, human chorionic gonadotrophin (hCG) being the most commonly used one. In the following 24 to 48 hour period after the hCG administration, approximately 85% of the mares will ovulate. The use of hCG should be limited to 2 injections per breeding season because it can be recognized by the immune system and lead to antibody formation. Deslorelin is another drug that can be used. It is a small molecule very similar to endogenous GnRH, which, after repeated use is less likely to cause antibody formation in comparison to hCG. Large amounts of LH will be released over a 6 day period after implantation of deslorelin. A 2.2 mg implant will effectively induce ovulation approximately 40 hours after

administration, as long as the mare is in estrus with a follicle ≥ 30 mm with uterine edema and relaxed cervix. When using hCG ovulation should take place in approximately 36 hours. One injection is needed when the follicle ≥ 35 mm for hCG or ≥ 30 mm for deslorelin or histrelin. Deslorelin is more beneficial to use compared to hCG since it is more stable, and it can be used repeatedly without developing refractoriness. The drawback of deslorelin is its costs, which is approximately 5 times that of hCG. (MC GREGOR ARGO, 2010, PYCOCK, 2008, SMITH, 2007)

In order to time the cycle, we can either shorten the diestrus period ("short cycle"), or we can postpone estrus. In order to shorten the cycle, prostaglandins are used. PGF₂alpha in the non-pregnant uterus is what causes the CL to regress in the mare. Prostaglandin should not be given if a large follicle is present. "The average mare ovulates approximately 7-10 days after prostaglandin treatment, however the range is large (as early as 2 days or as long as 10+ days) and depends on the size of the largest follicle at the time of PGF treatment."

(MACPHERSON, 2010). If the mare does not return to estrus within 5 days, ultrasound should be performed to make sure that the mare is in dioestrus, to estimate the age of the most recent CL, and to measure the diameter of the largest follicles present. If the mare is in dioestrus, luteolysis can be induced by PG analogues as long as the CL is more than 5 days old. After day 14, the CL should be regressing anyway. After the prostaglandin administration, ovulation will usually occur 8 to 10 days later. To postpone the estrus we use progestins. The CL in the mare produces progesterone. It takes her out of heat and it also maintains pregnancy. (BRINSKO, et al., 2011, MC GREGOR ARGO, 2010, METCALF, 2005, SMITH, 2007)

1.3 The timing and procedure of insemination

The longevity of spermatozoa within the mare genital tract after natural breeding is very variable, and it can last from only 24 hours up to seven days. The fertilizing capacity of most stallions is retained for at least two days. However, it is important to be aware of the different storage methods since these seem to have the most influence upon the timing of insemination relative to ovulation. The different storage methods are fresh non-cooled semen, cooled semen and frozen semen.

Prior to insemination it is important to check that the mare has a passport or identity document, and that the documentation accompanying the semen is checked as well as the health checks of the stallion. For imported semen from overseas the stallion must be seronegative for equine arteritis virus. Following each shipment there should be information on date and time of collection, motility, concentration, type and ratio of extender used. It is important to bandage the mare's tail and tie it out of the perineal region. Thereafter, the vulva

and perineal area should be thoroughly cleansed and dried. It is incredibly important that it is completely dry in order to avoid the growth of bacteria and fungi. If cooled semen is used, the container should be unopened until this stage, and the semen should not be warmed prior to insemination.

During insemination the catheter should be held by the index finger and inserted into the cervical canal. The catheter is then pushed forward, reaching the mid or cranial uterine body. Then the syringe should be carefully emptied, infusing the semen into the uterus. All instruments must be warmed beforehand in order to avoid temperature shock. Thereafter, a small amount of semen should be warmed to 37 °C and examined for any gross abnormalities and for progressive motility. At Doubletree we inseminated the mares from the end of April till late June, so that the foaling season would take place the next spring and early summer. Any later foaling is not preferred due to the good pastures and nice temperatures for a newborn foal during the spring and early summer months. (AMANN, et al., 2002, BRINSKO, et al., 2011)

1.3.1 Cooled and transported semen and its practical aspects

Cooled and transported semen is used all over the world, making distances in the horse breeding industry much smaller. As long as the initial quality of the semen is good, it is stored properly and the insemination is not delayed beyond 24 hours, the cooling of extended semen for transport is generally successful. For some stallions the pregnancy rates can be good even if the semen has been stored for 48 to 72 hours. Before we can breed mares successfully with cooled semen, the mare owner, stallion manager and the veterinarian must cooperate well when coordinating the semen shipments with the timing of the mare's ovulation. The veterinarian must clarify several points with the stallion owner before shipping the semen. These include the cost of stallion collection and shipment, what days of the week the stallion is collected, and the number of days notice the stallion manager needs before shipment. It is important to know the longevity of the semen in the tank, the first-cycle conception rate of the stallion, the method of air transport used, and the number of times the mare can be bred in case she does not conceive. The breed registry requirements and timing of post-insemination pregnancy examinations must be established. There is a tendency of the first cycle conception rates to be slightly lower with cooled shipped semen compared to natural breeding or artificial insemination using fresh semen. The costs are usually higher and the breeding management more intensive using cooled shipped semen. While the mare is in heat she should be examined daily and bred within 24 hours of ovulation. If a stallion is available at the facility where the mare is at, this may decrease the number of times she will need to be examined since heat can be more easily detected with teasing. The semen quality is

of paramount importance; stallions with high inherent fertility have a much higher conception rate than those with lower fertility. The handling of the semen is another critical point, as failure to prepare it correctly as well as poor subsequent handling of the mare can make the process very disappointing. The timing of breeding with the ovulation can be very difficult if the stallion is collected only 3 times a week. Hormones are often necessary in order to be able to time everything to fit. The semen quality must be adequate, with a minimum of 500 million motile sperm with at least 30% of them being progressively motile. For each time that the mare is bred with cooled semen, the semen should be warmed for a minimum of 3 minutes then carefully examined. (AMANN, et al., 2002, BRINSKO, et al., 2011)

1.3.2 Insemination with cooled shipped semen

The timing of insemination with cooled semen is more critical than that of fresh semen. It is not desirable to inseminate with cooled semen every second day as we can with fresh semen. Firstly, it could be extremely expensive to get semen shipped every other day. Secondly, the longevity of the cooled semen is less than the fresh semen, so more close monitoring between insemination and ovulation is required. The maximal pregnancy rates have been achieved if insemination occurs 0 to 24 hours prior to ovulation. Based on several studies, it is likely that the semen from about 70 to 80% of stallions survive the process of cooling and storage. The first cycle pregnancy rates of mares inseminated with fresh, cooled or frozen semen were 76, 65 and 56 %, allowing a comparison of the fertility of the fresh, cooled and frozen semen of a given stallion. There is approximately 10 % point decrease in fertility from fresh to cooled and from cooled to frozen semen. After 24 hours of storage, one should anticipate in approximately 20 to 30 % point drop in sperm motility. The semen that has been stored for 24 hours and then rewarmed has a motility of about 40 to 50 %. Even though the motility is good, the fertility can still be depressed. Other important factors to take into consideration are the mare management and the inability of the breeder and veterinarian to properly prepare the insemination.

Because the optimal time for insemination with chilled semen is in the 24 hours leading up to ovulation, it is important to accurately predict the time of ovulation. The time interval is shorter in chilled semen than fresh semen or natural service. The ideal time for breeding is shortly before ovulation, so the maximal fertility for most stallions using cooled-shipped or fresh semen can be achieved within 24-48 hours before ovulation or within 6-8 hours after ovulation. Typically, the cooled-shipped semen is 24 hours old at arrival, so ovulation should occur within 24 hours after insemination. This can be achieved by monitoring the mare until she has a large dominant follicle (>35mm) and uterine edema. The administration of an ovulation-inducing agent at the time when the semen is collected and

shipped will also help. This will result in ovulation of most mares within approximately 12 hours following insemination. Usually the two doses of semen are shipped, and the second dose can be inseminated 24 hours after the first one.

Before insemination, the chilled semen must be evaluated immediately before insemination using a warm-stage (37°C) microscope. More than 40% of the spermatozoa should be progressively motile. The mare should be re-scanned before insemination in order to evacuate feces, ensure the persistence of a dominant follicle and make sure there is no free fluid accumulated in the uterine lumen. It is essential to clean the perineum properly and apply a sterile non-spermicidal lubricant before introducing the insemination catheter into the uterine body per vagina. 24 hours after insemination the mare should be checked for any persistent fluid in the uterine lumen. In case of fluid accumulation, she should be treated with oxytocin or a combination of oxytocin, antibiotics and saline. This should be repeated until there is no free fluid remaining. In case of no ovulation within 36 hours of insemination, the mare should be bred again. (AMANN, et al., 2002, BRINSKO, et al., 2011, PYCOCK, 2008)

1.3.3 The insemination procedure using cooled equine semen

The procedure of breeding a mare with cooled semen includes these steps. First the mare needs to be prepared for breeding. Then the shipping container should be opened, the chilled semen carefully removed and gently mixed. The semen should be aspirated gently into a syringe and attached to an insemination pipette. The mare is then inseminated by infusion of the semen into the mare's uterus. Usually it is sufficient to deposit the semen in the uterine body, but if the semen quality is poor or the mare is subfertile a deep horn insemination is beneficial. From the extended semen a small aliquot should be warmed to 37°C in order to assess the sperm motility and record it for quality control purposes. In case of poor sperm motility, inquiries must be made to determine if this was unexpected. If this happens on several shipments and the mare fails to conceive on repeated breedings, the stallion may be the problem. He may be incapable of producing sperm that can survive the cooling and transportation process. If this is the case, the use of a different semen extender or dilution ratio, or the centrifugation and removal of excess seminal plasma should be tried, in order to improve the livability of the sperm from that particular stallion. A difference from the insemination procedure in Europe compared to the US is that in Europe they prewarm the chilled semen to body temperature prior to insemination. In the United States however, the chilled semen is placed directly into the uterus as soon as possible after opening of the shipping container. Since no detrimental effect to the pregnancy rate by this practice has yet been proven, the US breeders do not see any reason to prewarm the chilled semen before insemination. (BRINSKO, et al., 2011)

1.3.4 Breed allowances

Not all breeds allow the use of transported semen. One of the most recent breeds that allows the use of cooled transported semen is the American Quarter Horse Association. Others are the Arabians, the Morgans, and the Paint Horse Associations. The Thoroughbred is one of the major breed registries that still does not allow the use of transported semen. Even if it is allowed for certain breeds to use cooled semen, some breeders still choose to use fresh semen due to the increased cost of veterinary care and mare management with cooled semen. “To insure full compliance with the breed registry involved, the breed registry should be contacted prior to becoming involved in breeding with transported semen, so that all requirements for registration of foals can be met.” (BRINSKO et al., 2000) (AURICH, 2006)

1.3.5 The use of artificial insemination with frozen thawed semen

When using frozen semen it is important to store it in appropriate storage containers. The advantage of using frozen semen is that it is readily available all the time, so it is not necessary to have a stallion available when the mare is ready to breed. Frozen-thawed semen should be placed in the uterus within 12 hours before ovulation till 6-8 hours after ovulation. If multiple doses of frozen-thawed semen are available, it is convenient to inseminate at 24 hours and 40-42 hours after administering an ovulatory agent. This way it is only necessary to examine the mare 2-3 times after administration of an ovulatory agent. The first dose of semen will be in the uterus within 12 hours before ovulation, while the second dose will be there within 6 hours post ovulation. In case there is only one dose of frozen-thawed semen available, the mare should be bred in the post-ovulatory period. She should be examined every 6 hours by transrectal palpation and ultrasound, beginning 24 hours after the ovulation-inducing agent has been administered and until ovulation is detected. This ensures that the semen will be placed in the uterus within 6-8 hours following ovulation. (AMANN, et al., 2002, BRINSKO, et al., 2011)



Collection of a stallion, Perfect Sailer, at Doubletree Horse Farm. A mare in heat is being used as a teasing mare

1.3.6 Practical experiences

Doubletree Horse Farm breeds Quarter Horses and they raise between 10-15 foals a year, in addition to breeding brood mares for clients. I was involved in the entire program from breeding to foaling. The breeding season starts in April, and the days consist of taking care of foal and mare, as well as following the estrous cycle of the mares to keep track of when they can be rebred. We did ultrasound examinations to check the mares for signs of estrus, including the presence of follicles and their sizes. For each brood mare we have sheets to follow up approximately how big her follicle is when she ovulates, so it is easier to evaluate the following years and more accurate to predict so we do not miss an opportunity to breed her.

Ultrasounding of a mare at Doubletree Horse Farm to check the follicular size prior to breeding



Other important parameters to evaluate with the ultrasound are the presence of any fluid in the uterus, which may be a sign of infection. “There are several conditions or circumstances that could lead to fluid accumulation. Some of these include (1) inability if the uterus to contract because of age or uterine position, (2) cervical tightness or fibrosis, and (3) poor perineal conformation or cervical incompetence leading to urine accumulation.” (SAMPER, 2008) In these cases it is advisable to address and treat the uterine problem prior to breeding. “It is well established that a high percentage of mares that do not become pregnant have some degree of uterine contamination.” (SAMPER, 2008) Therefore it is good practice to proceed with uterine culture and cytology in these problem mares. Based on the results of the these tests, we proceed with uterine lavage and intrauterine or systemic administration of antibiotics. Some mares are having trouble with fluid in their uterus for

unknown reasons, and if they are having difficulties to conceive, the desire to breed them should be reevaluated. I have seen examples of mares being bred while having problems with fluid in the uterus, from the owner's wish to breed her, and they failed several times to conceive. In such instances it is preferable to treat the uterine problems and let her rest until her next cycle starts. She should then be checked with ultrasound for any fluids in the uterus again prior to breeding.

Using the ultrasound is also important in order to detect cysts in the ovaries and/or in the uterus. These may be harmless, but in many cases these mares are having a hard time to become pregnant. Detection of tumors is another important aspect of examining the mare using ultrasound. Granulosa cell tumors and theca cell tumors can develop in the ovaries. The granulosa cell tumor should be suspected if one of the ovaries is enlarged, while the other is normal sized. A parovarian cyst can be found next to the ovaries. These are not uncommon, but they are not thought to interfere with fertility. (SAMPER, 2008)

1.3.7 Costs and follow up

Artificial insemination is not a cheap alternative to natural breeding, and it requires a good follow up by both the owner and the veterinarian with a high degree of technical input. This is important to notify the owner about, as well as having a good communication with the mare and stallion owners. Another important issue considering the use of AI is the transmission of diseases. Therefore, it is extremely important to pay strict attention to the hygiene and to carry out good health precautions. Documents with information about the stallion, the collection centre, collection date, shipment date and information about the semen quality and number of sperm sent should follow each shipment of fresh or chilled semen. (BRINSKO, et al., 2011)

1.3.8 Post breeding mare management

Post breeding management of the mare is important and it includes the monitoring of ovulation either daily or every other day. If no ovulation is detected, the mare may need to be re-inseminated, in order to make sure that there are viable sperm within the oviduct at the time of ovulation. It is important to evaluate the mare for pathological changes like anovulatory hemorrhagic follicles, increased uterine fluid, and other evidence of persistent postmating endometritis. There is a physiologic endometritis occurring post breeding, which should resolve within 12-24 hours. In case this clearance fails, it is important to address the problem quickly in order to prevent infertility. If the mare has a history of post-mating endometritis she should be followed up more intensely with uterine lavage and tocolytic drugs.

There are several factors influencing the interaction between the uterus and the inseminate. These include the volume and concentration of the inseminate, the semen extender, the numbers of dead sperm, and the seminal plasma. It has been showed that the intensity of the reaction depends on the concentration and/or volume of the inseminate. So concentrated semen, e.g. frozen semen, induces a stronger inflammatory reaction in the uterus than what fresh or extended semen does. As long as the sperm concentration is above $25 \times 10^6/\text{mL}$ the volume of the inseminate is not a problem. It is important that the semen is not over extended, leading to low sperm concentration. This might lead to cervical reflux if large volumes are inseminated, again leading to a reduced fertility. Older maiden mares are often more prone to post-breeding endometritis. Common in these mares is the uterine fluid, as a result of an abnormally tight cervix that fails to relax properly during oestrus. This causes the fluid to accumulate in the lumen, which again is negative for bacterial growth and the presence of neutrophils. After breeding, this fluid accumulation is aggravated due to impaired myometrial contraction compounded by the tight cervix, as well as a poor lymphatic drainage. This is an important point to inform the mare's owner about. If the uterus is examined by ultrasound 12 to 24 hours after insemination, it often shows collections of fluid. In order for pregnancy to occur, these fluids must be removed, preferably by oxytocin. Intrauterine antibiotics can be beneficial in certain cases. The next examination will be the pregnancy diagnosis, taking place 14 to 15 days post breeding by ultrasonography. There are several reasons for this ultrasound examination, this article underlying some of the main factors: "Mares bred with cooled transported semen should be examined for pregnancy using transrectal ultrasonography 14-15 days after ovulation. This accomplishes a number of important functions. First, it allows early detection of pregnancy. Second, it helps establish if the conceptus and uterus appear normal for the stage of gestation. Third, it provides adequate time to contact the stallion manager and arrange for semen at the next estrus if the mare has failed to become pregnant. Fourth, it allows early contact with the stallion manager to inform them of the breeding results so that they can monitor pregnancy rates. And finally, it allows detection of twins before they become fixed in the uterus." (BRINSKO, et al., 2000) (AMANN, et al., 2002, SAMPER, 2008)



Brood mares and their foals out in the pasture in Delta, Colorado

1.3.8.1 Fresh semen

Storage of fresh semen can last for up to 10 minutes when it is undiluted, but ideally it should be inseminated immediately after collection. If it is to be stored for longer than 10 minutes, it should be diluted with an extender. The semen should be centrifuged in order to remove the seminal plasma if it is to be stored between 2 and 6 hours. This will delay the capacitation process. It is important to keep the semen stored in room temperature. A thermos may be beneficial to keep an even temperature of 18-22 C, and it must be airtight and lightproof.

2 Technique of semen cooling and freezing

2.1 Cooled and transported semen

2.1.1 The use of extender

In order to be able to maintain the viability of stallion spermatozoa, it is absolutely essential to dilute the seminal plasma. The seminal plasma is generally not a good extender for the spermatozoa on its own, it needs to be extended with an appropriate diluent. Therefore, when the semen is to be shipped and has to be stored for a while, it is extremely important that it is being kept in a good extender. These extenders contain protective ingredients that permit the sperm to survive outside of the reproductive tract. Then the life of the spermatozoa is extended, and this way the volume of the inseminate can be increased. A proper evaluation of the sperm motility is possible if it is extended. If stored for more than 6 hours, the semen must be cooled in order to prevent the spermatozoa of losing too much energy. Dilute the gel-free ejaculate with a suitable extender at 37°C, and check the motility using a light

microscope. “An ejaculate should be collected, diluted 1:2 with an extender such as heated-cooled skim milk, placed in an Equitainer, then examined at 12 hour intervals for sperm motility. Because one usually packs at least 500 million progressively motile sperm per insemination dose, dilution rates will vary (1:1 to 1:6) according to the density of the semen collected.” (PYCOCK, 2008).

2.1.2 Ingredients of the extender

Milk and egg yolk contain protective lipoproteins that protects the sperm against cold shock by stabilizing the cellular membranes. Glucose is a good source of energy for the sperm, and antibiotics are added to decrease the growth of bacteria and transmission of pathogenic organisms. It is important that the antibiotics are present in the extender when the semen is added, thus avoiding damage to the spermatozoa with a too high concentrations of antibiotics. The pH and the osmotic pressure are adjusted to maximize the sperm survival. The pH should be between 6.5 and 7.2, and the osmolarity should be between 300 and 400 mOsm/L. Other important ingredients in the extender include a proper balance of mineral elements (i.e. electrolytes), a proper nutrient combination, neutralization of toxic products produced by the spermatozoa, protection against temperature change, and stabilization of enzyme systems and integrity of membranes. Egg yolk, milk, milk by-products and chemicals have been used as extenders to regulate the osmolarity and/or pH, the ratio of skim milk-glucose being at least 3:1. The milk-based extenders are most commonly used worldwide. (AMANN, et al., 2002; AURICH, 2006, BRINSKO, et al., 2011, BACKMAN, et al., 2004, HEISE, et al., 2011)

The ejaculate must be diluted to a concentration of about 25 million motile spermatozoa per milliliter. A too high concentration of the seminal plasma is deleterious to the cooling and storage of the extended semen. By diluting the plasma with skim milk-extender, this problem can be reduced. The antibiotics that are used in extenders are most commonly amikacin sulphate and potassium penicillin G, ticarcillin and ceftiofur. It is very important that the antibiotics are not detrimental to the sperm function. The main goal of the antibiotic is to eliminate all bacterial growth yet not hamper the sperm viability. The semen should ideally be mixed with a prewarmed (37°C) extender within 2 to 5 minutes after ejaculation. For immediate insemination, a minimum ratio of 1:1 of extender to semen is recommended. Greater dilution (higher extender to semen ratio) is necessary if the semen is stored for 2 hours or longer before insemination. To maximize the sperm survival in vitro, a final concentration of 25 to 50 million sperm/mL is desirable. The final seminal plasma concentration in the extended semen should be less than 20%, so a minimum of 1:4 dilution (semen:extender) should be obtained in order to maximize the sperm survival. The seminal

plasma of some stallions has detrimental effect on the sperm viability, even at concentrations as low as 5 to 10 % (volume/volume [v/v]) in the extended semen. If this is the case, it may be necessary to centrifuge the semen after its initial dilution with extender, followed by removal of virtually all the seminal plasma. Thereafter, the pellet of sperm can be resuspended in extender preloaded with sperm-free seminal plasma (5 to 10 % [v/v]) from a fertile stallion with good quality seminal plasma. Prior to donation, it is important to ensure that the seminal plasma of the donor is free of organisms that may cause transmissible diseases, such as EIA, equine viral arteritis, or CEM. (AMANN, et al., 2002; BRINSKO, et al., 2011, BACKMAN, et al., 2004, HEISE, et al., 2011)

Since the spermatozoa deteriorate quickly after collection unless they are extended, this is crucial to do as soon as possible. When the semen is to be inseminated immediately, it should be kept at room temperature once it has been extended. The cooling should be initiated immediately after being extended if the semen is to be stored for 1 to 2 days before breeding. The purpose of cooling the spermatozoa is to slow down the cellular metabolism in order to prevent deterioration. The more rapidly the cooling can be done while avoiding cold shock, the better. What determines the cooling rate of extended semen depend on the volume and initial temperature of the sperm suspension. Cooling of the spermatozoa can, if done improperly, affect the capability to capacitate, acrosome react and fertilize an ovum. For this reason, it is very important that this is done correctly. In order to be able to fertilize an ovum, the spermatozoa must be able to retain the metabolic processes for energy production, and the progressive motility must be retained. The proteins that are necessary to survive in the female reproductive tract must retain, as do the enzymes that are essential for the sperm to penetrate the outer structures of the ovum, and proper distribution of plasma membrane lipid and protein is important in order to permit a timely acrosomal reaction, binding and fusion of the ovum.

2.1.3 Cold Shock

Cold shock is the damage to the spermatozoa as a result of too rapid cooling from room temperature to 5°C. It includes partially irreversible damages, which is characterized by abnormal patterns of motility (circular or backwards movements), rapid loss of motility, acrosomal damage, damage to the plasma membrane, reduced metabolism and loss of intracellular components. The cellular injury can be both direct and indirect, the direct one affecting cellular structures (like rupturing membranes), and the indirect ones affecting cellular functions. The damage is partly caused by cooling from liquid-crystalline to gel state. By including extender and a slow and careful cooling this damage can be minimized. Passive cooling systems generate variable cooling rates, so they become progressively slower as the

target temperature is approached. Other factors influencing the cooling rate are the environmental temperature, initial temperature of semen, and the volume of extended semen that is being cooled. The initial cooling rate should be $-0.3^{\circ}\text{C} / \text{min}$ in order to maximize the sperm viability, and this can be achieved using an Equitainer. The spermatozoa from stallions are most susceptible to cold shock induced by rapid cooling from 19 to 8°C , while from 37 to 19°C and from 8 to 4°C it can be cooled rapidly without the danger of causing cold shock. Accordingly, the cooling rates from 19 to 8°C should be -0.05°C to $0.17^{\circ}\text{C} / \text{min}$. Most protective additives contain lipids or lipophilic molecules, and additives such as calcium and magnesium protect the spermatozoa by binding ions and reducing ion leakage into the spermatozoa during membrane transitions. Egg yolk is common in stallion semen extenders as a lipid additive. The concentration is usually 2 to 20%. The phospholipids in the egg yolk stabilize the sperm membrane, and extenders containing skim milk are commonly used to protect the spermatozoa from cold shock. (AMANN, et al., 2002, BRINSKO, et al, 2011)

2.1.4 Shipment

Before being loaded into semen shipment containers, the semen is usually packaged in plastic bags or aluminum plastic syringes. It is the type of container that decides what package type should be used. Pertinent information regarding the stallion identification, semen collection date, and mare to be inseminated should be labeled and easy to identify. Any additional information should be available on an accompanying semen transport form. Before arranging transportation of semen, the health authorities that are responsible for importation of semen should be contacted to ensure that the requirements are met. Some countries require an extensive health screening before semen can be collected for preservation. These may include serology testing, culturing of reproductive tract or semen, inspection of premises and animals by regulatory officials, and vaccination. (AMANN, et al., 2002, BRINSKO, et al., 2011, LOOMIS, 2005)

2.1.5 Semen evaluation

In order to determine the most accurate insemination dose, semen-cooling trials for individual stallions should be conducted. The semen is diluted in an extender and stored for 24 hours, before it is remixed and an aliquot is warmed to 37°C . After 15 minutes of warming, the sperm motility is evaluated, and the percentage of progressively motile spermatozoa is obtained. This serves as a guideline to ensure that future shipments provide a minimum of 500 million progressively motile spermatozoa after 24 hours of cooling. As an example, if the progressive sperm motility is 50% after 24 hours of cooling, 1 billion total sperm would need to be prepared for shipment in order to ensure that the insemination dose

for breeding a mare contains 500 million progressively motile spermatozoa. If there is a demand for 48-hour cooled semen, the same procedure can be done on semen cooled for 48 hours. Reference laboratories can conduct more extensive studies for cooled semen if it is justified. These methods include objective measurement of sperm motion, membrane integrity, chromatin quality, intact acrosomes, and morphology after cooled storage. It is advisable to test the storage ability of the stallion before shipping semen from that stallion, as some stallions have semen that transports well in cooled state while others have not. "The semen is appropriately diluted in extender, and then cooled for 24 hours. Spermatozoal motility is evaluated after warming to 37°C, and the percentage of progressively motile sperm following storage is used to help ensure that shipments will provide a minimum of 500 million progressively motile sperm after 24 hours of cooling." (BRINSKO, et al., 2000). Disadvantages of this method are that it only measures one sperm function, it depends a lot on the experience of the person evaluating it, and a high percentage of motile spermatozoa are not necessarily correlated with fertility. The following table, based on embryo recovery, is a proof that the fertility was different from the different stallions, even though the motility from the five stallions was similar. (AMANN, et al., 2002, BALL, 2004, BRINSKO, et al., 2000, BRINSKO et al., 2011, HECKENBICHLER, et al., 2010, KUBIAK, 2013)

Table 4: The percentage of motile spermatozoa and corresponding fertility of spermatozoa in semen from five stallions after spermatozoa were frozen and thawed. (AMANN, 2002)

Stallion	Motility (%)	No. mares	Embryo recovery (%)
A	43	19	74
B	43	18	78
C	42	18	28
D	38	18	28
E	34	19	16

The fertility outcome depends just as much on the spermatozoa as it does on the proper management of mares, proper timing of the insemination during the mare's cycle, and proper techniques for insemination. To evaluate the cooled semen it is important to count the spermatozoa first. Then you estimate the motility and determine the volume of extender and raw semen needed for shipment by this formula: Volume of raw semen = 1,000 million (concentration) X (progressive motility) to calculate the volume of extender and raw semen. The formula should help determine the volume of semen needed to provide 1 billion progressively motile spermatozoa. Thereafter, the raw semen should be added to provide at

least 3:1 dilution and a concentration of approximately 25 million progressively motile spermatozoa per milliliter. If the volume of raw semen contains 1 billion progressively motile spermatozoa and enough extender is added to provide a final volume of 40 ml, then the concentration always is 25 million motile spermatozoa per milliliter. However, it may be necessary with a final volume that is greater than 40 ml in order to provide at least a 3:1 dilution ratio. (AMANN, et al., 2002, BRINSKO, et al., 2000, BRINSKO et al., 2011, HECKENBICHLER, et al., 2010, KUBIAK, 2013)

2.1.6 Induction of ovulation

There are two protocols commonly used for induction of ovulation using chilled shipped semen. One of them includes IV injection of hCG when the follicle exceeds 35 mm in diameter with uterine oedema and a soft cervix. 90% of the mares will then ovulate within 48 hours. The other option is a subcutaneous implantation or IM injection of deslorelin, a GnRH agonist, can be used when the follicle exceeds 30 mm and uterine edema is noted. Within 36-42 hours 90 % of the mares should have ovulated. “For chilled semen, conception rates are greatest when insemination is within the 24 h preceding ovulation. The relatively short life (12 h) of frozen semen requires that mares are inseminated immediately before (within 6 h) or after (within 4 h) ovulation. If 2 frozen semen doses are available, fixed-time inseminations at 24 h and 36-40 h post hCG treatment will reduce scanning requirements.” (MC GREGOR ARGO, 2010) (SMITH, 2007)

2.2 Frozen semen

2.2.1 Packaging of frozen semen

The packaging is either done in 5 mL or 0.5 mL straws. Generally, the 5 mL straws contain about 600 to 800 million total spermatozoa, and one straw is used per breeding. The 0.5 ml straws contain 50 to 100 million, and usually 6 to 8 straws are used in one insemination dose. The straws must be labeled with pertinent data such as stallion identification, processing date, breed registration number, and laboratory identification. To load the straw, you can either use automated straw filler or they can be loaded manually using a manual mouth pipette. There must be an air bubble in the center of each straw before freezing. The straws should be frozen over liquid nitrogen vapor in a Styrofoam cooler. (AMANN, et al., 2002, BRINSKO, et al., 2011, KUBIAK, 2013)

2.2.2 The storing and thawing process

After submerging the semen in liquid nitrogen, they are contained in special storage tanks called dewars in order to maintain the sperm in a dormant state. The 5 mL straws are

placed directly into the plastic goblets, which again are attached to canes before they are placed into canisters. The goblets can be color-coded in order to make it easier to access the correct straw easily without having to go through so many of them and thereby expose them to high temperatures. After freezing, the post-thaw motility of the spermatozoa should be evaluated. One straw should be quickly removed from the goblet and plunged into a 37°C water bath for 30 seconds for a 0.5 ml straw, and for 45 seconds in a 50°C water bath for a 2.5 ml or 5.0 ml straw. The straw must be wiped dry and cut off an end. The open end should be put in 10 ml of warmed extender, like the E-Z Mixin – BF extender. Then the remaining end of the straw should be cut, so that it will drain into the cylinder. It must be gently mixed and allowed to sit in the incubator at 37°C for 10 minutes prior to motility evaluation, which should be estimated at 10 and 30 minutes after thawing. 10 minutes after thawing, the percentage of progressively motile spermatozoa should be $\geq 30\%$. (AMANN, et al., 2002, BRINSKO, et al., 2011, KUBIAK, 2013, SAMPER, 2001)

2.2.3 The freezing process

Before freezing, the straws must be hermetically sealed, with an air space present in the straws to permit expansion of the content. The air space is shaken to the middle of the straw before sealing in order to reduce the likelihood of liquid nitrogen access into the straw lumen. The freezing extender should maintain the viability of the spermatozoa during cooling from room temperature to 5°C, as well as during freezing and thawing. For protection of the spermatozoa during cooling to 5°C, we use lipid sources such as egg yolk or milk lipoproteins, and for freezing cryoprotectants such as glycerol and sugars are added while cooling below 5°C. Two commonly used freezing extenders are lactose-EDTA, which contains no milk and does not require cooling of the semen prior to freezing, and the French extender that does contain milk and requires cooling for 2 hours prior to packaging and freezing. The freezing extenders must be stored frozen, and then just prior to use must be thawed and warmed to 22°C. (AMANN, et al., 2002, BRINSKO, et al., 2011, KUBIAK, 2013)

2.2.4 Factors affecting the fertility of frozen-thawed semen

The single greatest factor is without any doubt the stallion. About 35% of them are good freezers, 45% are moderate freezers, while 20 % are considered to be unsatisfactory freezers. Even though the fertility is poorly correlated with post-thaw motility, this is the only practical tool for assessing the freezability of the semen and predicting the fertility. The freezability or fertility is affected by several factors. One of these is the centrifugation, where semen from most stallions can withstand very significant g-forces, showing that up to 2400 x

g not is detrimental. Some stallions require the use of a special cushion fluid during centrifugation. The freezing and thawing rate can have significant influence on the freezability. All of the commercially available freezing extenders have been pretested for their optimal freezing and thawing rate, so if a stallion does not pass a test freeze, one can try to alternate freezing and thawing rates with the various freezing extenders. The centrifugation and freezing extenders can both have significant influence on the freezability of a given stallion. In order to determine which combination provides the best post-thaw motility for a given stallion, several centrifugation and freezing extenders combinations should be used in a test freeze. There are some factors that have not shown to affect the freezability or fertility, and these are the packaging method, the concentration, and the season of the year. (AMANN, et al., 2002, BRINSKO, et al., 2011, KUBIAK, 2013)

2.2.5 Test freeze and dosages of semen

The test freeze serves several purposes and is an essential part of the freezing process. The purpose is to determine which combination of the following factors that will provide the best post-thaw motility: centrifugation, centrifugation extender, freezing extender, freezing rate, or thawing rate. In order to test the frozen semen, breeding mares must be used. There is a fertility guarantee made by the Pacific International Genetics saying that the owner has 2 reproductively healthy mares. If at least 1 pregnancy is achieved within 2 cycles on either mare, the semen is considered to be fertile. If no pregnancy is obtained, the mares should be rebred from the 2nd best protocol. There is no set standard dose, but the most commonly used standards are 800×10^6 total sperm cells in North America, and $250-300 \times 10^6$ progressively motile sperm cells in Europe. The number of straws used per dose varies a lot from place to place, and as few as 1 straw or as many as 16 straws per dose can be used. (AMANN, et al., 2002, BRINSKO, et al., 2011, KUBIAK, 2013)

2.2.6 The quality of frozen semen and factors affecting it

In frozen equine semen the volume should be 10-200 mL, the concentration 50-400 ($\times 10^6$), the total spermatozoa 3-24 ($\times 10^9$), and the progressive motility 40-90%. Measures used for sperm quality is usually the progressive motility in % and the morphology. What does not affect the sperm quality is the age and testicular size, the season, the frequency of ejaculation, and teasing with mares. What can affect the quality negatively is the cryptorchidism, which will lead to a decreased motility and morphology; hemospermia, which will make the spermatozoa infertile, urospermia; which will depress the motility severely; and in cases of increased extragonadal reserves, the motility will be decreased. Stallions that have been rested often need a clean-out 3 to 4 times in order to get rid of the extragonadal sperm

reserves. During the spring, summer or fall the stallion should be collected every 2-4 days. The best time to do the freezing is between 5 and 15 years of age. In cases of older stallions, testicular degeneration occurs, leading to a decrease of total sperm, initial motility, concentration and morphology. In cases of too young stallions (<3 years old), there is a lower sperm output. (AMANN, 2002, BRINSKO, 2011, KUBIAK, 2013)

2.2.7 Freezing extenders and liquid nitrogen

Most of the extenders used for freezing consist of milk or egg yolk as protein sources, sugars as energy sources, electrolytes as buffers, glycerol as cryoprotectant, and antibiotics. In order to calculate the appropriate amount of freezing extender, this equation can be used: mL freezing extender = mL extended semen x 0.0875. So, for a tube containing 24 mL of extended semen would need: 24 mL x 0.0875 = 2.1 mL of freezing extender. In order to maintain the liquid nitrogen tanks properly, they have to be stored in a well ventilated and dry room. They should be put on a dollie so that they do not stand directly on the floor. Daily inspection is necessary, making sure that the tanks do not feel cold and that they have no condensation. The liquid nitrogen levels should be checked weekly, and the tanks must be filled when the level drops to half capacity. They should never drop below 5 cm. Regular inspection of the neck cork for any damage should be carried out. (LOOMIS, 2001)

2.2.8 Shipping of frozen semen

Shipment of frozen semen can take place either weeks or months prior to breeding, or when needed using reusable or disposable dry shippers. The reusable ones will maintain semen for up to 12 weeks, while the disposable ones will maintain semen for 4 days. The reusable dry shipper should be filled with liquid nitrogen overnight, internal sponges will soak up liquid nitrogen, and the remaining LN₂ should be poured back into the tank. The semen is then placed into a canister, and the dry shipper is placed into a protective shipping carton. Shipment can take place via courier or airline. A dry shipper must be charged 24 hours in advance of loading for shipment. (BRINSKO, et al., 2011, KUBIAK, 2013, LOOMIS, 2001)

2.2.9 The thawing procedure

The mare should be washed and ready for insemination before opening the container. Then the frozen semen containers should be as close as possible to the water bath. The lid of the container should be opened, and the tweezers should be precooled below the frost line in the neck of the container for 1 minute. Thereafter, the canister containing the straws is then lifted, to the level of the frost line. One straw should be removed quickly using tweezers or pliers into the water bath. Then the lid of the container should be closed. After 30 seconds in

the water bath, the straws should be removed and dried off with a clean paper towel. It is important to examine that the labeling is correct. The sealed end of the straw is cut off and the straws are out upside down into a 15 mL prewarmed test tube at 37°C. The other end is then cut and the straws empty into the test tube. Each straw will still contain a tiny amount of semen, so one straw should be tapped on a prewarmed slide and a prewarmed cover slip should be placed over the drop of semen. The remaining semen should be blown into the prewarmed test tube, using the mouth. The slide should be incubated for 5 minutes, and then examined for progressive motility. If it is poor (<25%), the slide should be returned to the incubator and reexamined after another 5 minutes. In cases of poor results, this should be confirmed with a second slide. The syringe and insemination pipette is attached, and then 5-10 mL of air is drawn up to make sure that all the semen will be evacuated during insemination. The end of the insemination pipette should be placed into the test tube and all of the semen drawn into the pipette. The mare should be inseminated immediately. The semen pipette should be covered with the gloved hand in order to minimize temperature loss. The pipette is passed through the cervix and the semen is deposited slowly into the uterine body. To help stimulate uterine contractions, massaging of the external cervical os and the anterior vaginal vault for a minute post-insemination. This will help propel the sperm towards the oviducts. It is important to avoid exposure to sunlight or inclement weather conditions. The semen should ideally be in the mare within one minute of thawing. It is not recommended to use frozen semen in breeding very early in the season when the follicle development is slow and erratic. Postovulatory breeding has shown excellent results provided that insemination happens within 6 hours of ovulation. (AMANN, et al., 2002, BRINSKO, et al., 2011, KUBIAK, 2013)

2.2.10 Cryopreservation

For each 10°C drop in temperature, the metabolism of the sperm cell decreases with 50%. Thus, when the semen is frozen and stored in liquid nitrogen at -196°C, the metabolism is nearly nonexistent. Accordingly, there is no waste products, lipid peroxidation or wearing out of the sperm cells as there would be in normal metabolic rate at body temperature. Cryopreservation of stallion spermatozoa has certain principles that need to be followed. First, the spermatozoa must be mixed with an appropriate extender, and then centrifuged in order to remove the seminal plasma and concentrate the spermatozoa. After centrifugation the spermatozoa must be resuspended in a freezing extender containing a cryoprotectant, cooled to -120°C and stored in liquid nitrogen at -196°C. There are several factors that determine the success in cryopreservation of stallion spermatozoa, including a complex series of interactions among extenders, cryoprotectants, and the cooling and warming rates. Several features that is

important to keep in mind also when using cryopreservation of equine semen so that successful fertilization can take place. These are the progressive motility, a normal metabolism, intact cellular membranes, the presence of acrosomal enzymes, intact surface-associated proteins responsible for sperm-egg interactions, and uninjured nucleoprotein. The cryopreserved sperm undergo tremendous stresses during the process of freezing, thawing and insemination, and the fact that sperm can survive these processes is amazing.

2.2.11 Cryoprotectants

There are penetrating and non-penetrating cryoprotectants. Among the penetrating ones, glycerol is the most commonly used cryoprotectant. Its properties include an increase in the volume of unfrozen channels of solvent, and it dilutes high concentration of salt. The non-penetrating cryoprotectants include lactose and lipoproteins, which are found in egg yolk. These are able to dehydrate the sperm cells and, accordingly, decrease the likelihood of large ice crystals forming within the sperm cells. A proper concentration of the cryoprotectant is essential, as a too high concentration is toxic while a too low concentration provides insufficient protection. (AMANN, et al., 2002, BRINSKO, et al., 2011)

2.2.12 The freezing procedure

Prior to freezing, the sperm concentration should be increased in order to be able to load the insemination doses into small packages, while reducing the percentage of seminal plasma contained in the resuspension medium. The Colorado State University laboratory uses E-Z Mixin – BF, and here the raw semen is extended to 50 million spermatozoa per mL. The freezing procedure includes several steps, beginning with collection and evaluation of the semen. The gel-free seminal volume, sperm concentration and the percentage of progressively motile spermatozoa are determined. Thereafter, the semen is diluted with a centrifugation extender, in order to minimize cellular injuries. The centrifugation medium can be either solutions of dried skimmilk and a sugar, or a combination of salts and sugars. Then centrifugation is done in order to remove seminal plasma and concentrate the sperm cells. After that there is a resuspension in the freezing extender that contains the cryoprotectants. These extenders generally consist of mixtures of egg yolk, milk, sugars, buffers, electrolytes, antibiotics, and a cryoprotectant such as glycerol. Then loading into straws, vapor cooling to -120°C, and plunging into liquid nitrogen at -196°C is done. The next step is evaluation of post-thaw motility from one straw, before storing in liquid nitrogen at -196°C. (AMANN, et al., 2002, BRINSKO, et al., 2011, KUBIAK, 2013)

When freezing spermatozoa, ice crystals begin to form, and the salt portion of it remain in the unfrozen parts of the sample. As the temperature decreases, the concentration of

salt in these channels keeps increasing. Even at -196°C , the channels remain with an extremely high salt concentration, and this is the only place where the spermatozoa can survive freezing. Accordingly, freezing semen with too high sperm concentration to reside in these channels will result in a lower percentage of spermatozoa surviving the freezing process. Before freezing, the sodium chloride concentration is 0.9%, while after freezing till -12°C it has increased 20 times. A too high salt concentration can dehydrate the spermatozoa. This will result in cell deformations, damage to the membrane structure, dislodgement of the membrane proteins, denaturation of proteins and disassembly of the cytoskeletal structures, all of which can be detrimental to the spermatozoa. In order to protect the spermatozoa during freezing and thawing, cryoprotectants must be added to the medium. They can be either penetrating or non-penetrating. Glycerol acts both intra- and extracellularly by protecting the cellular structures. The action of glycerol is to increase the volume of unfrozen channels of solvent and dilutes the high concentration of salts. Lactose and lipoproteins from the egg yolk are non-penetrating cryoprotectants, and they act only in the extracellular compartment. Their aim is to dehydrate the spermatozoa, thus reducing the probability to form large ice crystals within the cells. Too high concentrations of cryoprotectants are toxic to the spermatozoa, leading to lower fertility rates. The reasons for this can be multiple, but direct injury to the cell, cellular osmotic damage, and altered interactions between the spermatozoa and the female reproductive tract are possible solutions. "...during spermatogenesis the DNA of the cell is condensed in such a manner that genes cannot be expressed, hence no proteins can be made by the spermatozoon. This means the cell cannot repair itself from any cellular damage that occurs naturally or due to human interventions (semen handling, cooling or cryopreservation). Improper handling of spermatozoa can permanently damage spermatozoa, making them infertile" (GRAHAM, 2001) For this reason, the aim is to have a concentration of cryoprotection that maximizes the protection of the spermatozoa, yet minimizes its toxic effect. (AMANN, et al., 2002, GRAHAM, 2001)

2.2.13 Important considerations regarding frozen semen

In order for a spermatozoon to fertilize an oocyte, after freezing and thawing it must retain a minimum of four general attributes: 1) the metabolism to be able to produce energy, 2) progressive motility, 3) intact acrosome containing the functional enzymes necessary for penetration through the structures surrounding the oocyte, and 4) the proteins on the plasma membrane that are important for the survival of a spermatozoon within the female reproductive tract and for the attachment of the spermatozoon to the plasma membrane of the oocyte at fertilization. If any of these components are disrupted, the fertility will be reduced or even abolished. Sometimes the motile spermatozoa are not fertile, indicating that adequate

production of energy is present but other functions have been altered. Another factor that is important to take into consideration is that the volume of the sample and the surface area per unit volume affect the freezing rate of the sample. This means that larger volumes will freeze more slowly than smaller volumes at the same temperature. “As a generalization, the more rapid the cooling (freezing rate), the more rapid should be the warming (thawing rate). The ideal cooling and warming rates also are influenced by the concentration of ingredients in the extender, including the concentration of glycerol. Typically, 0.5-ml straws of stallion spermatozoa are warmed at 700°C per min by immersion in 37°C (98.6°F) water for 30 sec, or 4,000°C per min by immersion in 75°C (167°F) water for exactly 7 sec.” (AMANN, et al., 2002)

2.2.14 Insemination with frozen-thawed semen

The timing of the insemination is different in cooled shipped and frozen thawed semen. What determines the lifespan of the spermatozoa in the female reproductive tract is the maximum interval from insemination to ovulation that will provide maximal fertility. After entering the reproductive tract, the spermatozoa attach to the epithelial cells lining the oviduct. Based on recent studies, the spermatozoa that has been frozen and thawed does not interact to the epithelial lining as well as those in fresh semen. Mares inseminated with frozen thawed semen should be inseminated closer to ovulation than those bred with fresh semen. What is recommended is that mares bred with frozen semen should be inseminated from 0 to 24 hours prior to ovulation till 6 hours post ovulation. The quantity of the semen available is what determines the timing of the insemination. “..most practitioners use one of two protocols for breeding frozen semen mares: timed insemination at 24 and 40 hours after ovulation induction, or frequent palpation and ultrasonography with insemination occurring once or twice within 2 to 8 hours of ovulation.” (SCHMIDT, 2011)

2.2.15 Practical aspects

Before deciding what stallion to use, it is advisable to investigate the fertility of that stallion. This number of mares bred with frozen semen, the seasonal pregnancy rate and the pregnancy rate per cycle should be assessed. If there is no record of mares bred with that stallion, the number of sperm per dose and the post-thaw motility should be assessed. A contract should be read, fully understood and signed. A timeline must be set as of when the semen should be shipped. The veterinarian should perform ultrasound to determine the stage of cycle, the follicular size and pregnancy status. Ultrasound is usually required every 6 hours. Culture is strongly recommended, as well as biopsy and uterine treatment if required. Hormones should be utilized in order to properly time the cycle and ovulation. Before

insemination the veterinarian should thaw the semen and evaluate the post-thaw motility, before inseminating the mare. Ultrasound exam and treatment of any post-breeding endometritis is performed after insemination. It is important that the mare owner provides information of any known reproductive history of the mare, like the follicular size at ovulation, follicle growth rate, length of heat, and responsiveness to hormones. Most likely will the mare have to stay at the breeding facility in order to be able to conduct ultrasound exams every 6 hours for 24 to 48 hours. The mare might respond differently to the hormones than expected. The ovulation may happen prematurely, so the mare must be bred on a subsequent cycle instead. Or the mare might not ovulate within the expected time frame, making it necessary to ultrasound for a longer period of time. It is important to conduct the motility assessment after thawing each dose prior to insemination, and to follow the instructions thoroughly. Knowledge of the semen quality is essential, as dead semen or semen of poor quality should not be inseminated. Cases of poor results must always be confirmed by redoing them. (BRINSKO, et al., 2011, KUBIAK, 2013)

2.2.16 Common mistakes

One of the major causes for a low fertility associated with the use of frozen semen is the inseminator. The key factor in order to enable a successful, long-term storage of frozen equine spermatozoa is low temperature, which should not be warmer than -130°C at any time. It is the packaging system that will determine how quickly the temperature rises when the package is exposed to ambient conditions. No individual straws must be exposed to ambient conditions without additional protection. When removing straws for thawing, the canister should be raised no higher than absolutely necessary and the individual straws should be removed as quickly as possible. The damage caused by exposure of elevated temperature is additive. Keeping the tank reasonably full of liquid can minimize problems of damaging spermatozoa during transfer. Also, when transferring semen from one LN tank to another, the tank of which the semen is to be filled should be filled with LN before starting the transfer. A LN tank in which semen is stored should only be opened for these three reasons: 1) semen is being removed for breeding mares, 2) semen is being removed and placed in a shipper for shipment to clients or storage, or 3) to determine the level of LN in the storage tank. Unfortunately, there have been several occasions where the motility of the spermatozoa in the straws in the top goblets was inferior to those in the bottom goblets, these were where frozen semen has been stored at the farm. (AMANN, et al., 2002, KUBIAK, 2013)

2.2.17 Removing straws from the liquid nitrogen

Removing of the straws from a tank needs to be done in a certain way, starting by removing the insulated plug from the mouth of the LN tank. The canister containing the stallion's semen should then be located, and it must be lifted no higher than the frost line in the neck of the tank, and the cane containing desired straws should be located.

The upper end of the cane should be quickly grasped with a forceps, containing the desired straws and allow the canister to fall back into the tank. Then the top of the can should be identified with a tab indicating the ejaculate. The individual straw within the goblet should be grasped and removed, keeping the goblet within the neck of the tank. When the straw is held firmly in the forceps, it should be able to fall back into the canister. The straw should be removed with a forceps and immediately placed into a water bath maintained at 37° for thawing. Exactly 30 seconds must be allowed for thawing. Only one straw should be removed and thawed at a time. If two or more straws are placed into the thaw bath at the same time, they may freeze to one another and reduce the rate of thawing, which is undesirable. (LOOMIS, 2001)

2.3 Differences between semen quality and pregnancy rates in case of the three method of AI

The semen quality and evaluation of the semen is an important part of the reproductive veterinary service. Examining a single drop of semen in the light microscope provides good information about the motility of the ejaculated sperm. Also the cooled-shipped semen should be evaluated before it is being packaged and shipped. The veterinarian managing the mare to be inseminated should evaluate the semen for motility, morphology and adequate numbers. A standard dosage for fresh semen is 250 to 500 million progressively motile spermatozoa. For cooled-shipped semen the dosage is at least 1 billion progressively motile spermatozoa at the time of shipment, and at least 500 million at the time of insemination. The standard for frozen-thawed semen is 1 billion to 800 million total sperm/dose with a minimum of 30% motility after thawing. (AMANN, et al., 2002, BRINSKO, et al., 2011, KUBIAK, 2013)

2.3.1 Pregnancy rates

The expected pregnancy rate with chilled semen is 55-70 % and with frozen semen 35-50 % per cycle. At the end of the season the overall pregnancy rates varies between 50 and 90 %, the average being about 75%. Since obviously some mares loose the pregnancy, the live foal rate is about 65%.

2.4 Advantages of artificial insemination

There are several advantages in association with artificial insemination. The disease control is an essential part of it. The spreading of venereal diseases is more easily monitored during artificial insemination than in natural breeding circumstances. The amount of semen is less, so that the number of organisms is reduced below the number necessary to cause disease. The antibiotics in the extender will either kill or destroy the pathogenicity of the organisms, thereby minimizing the transmission of venereal diseases to the mare when the stallion serves as a carrier. Also, the transmission of potential pathogens from the mare to the stallion is reduced this way. The extenders contain supportive and protective factors for the sperm, so the pregnancy rates of some stallions may improve. There is less chance of injury to both the stallion and the mare, since the stallion is collected using a phantom mount or a gentle mare that accepts him. The use of an artificial vagina for semen collection allows scrutiny of the semen quality before insemination takes place. Accordingly, there is allowance for an early detection of problems that may adversely affect the fertility of the stallion. Another benefit is the size difference from between the mare and the stallion, which is not an issue when using AI. At each collection the semen can be evaluated so that minor changes in the semen quality can be immediately recognized. The formation and maturation of the spermatozoon requires about 57 days in the stallion. Otherwise, undetected reproductive problems can be identified if used in conjunction with an effective herd health program. Sometimes AI is falsely said to be the cause of low fertility, while in reality it only aided in pointing out the problems that pass undetected during natural service. The overuse of a stallion can be prevented, especially early in the breeding season. This is a common cause of subfertility or sterility, particularly in young stallions. Most mature stallions, from about 5-6 years of age, can be used daily, twice or occasionally three times per day without reducing the sperm output below the minimum number needed for a maximum fertility. Other stallions are more sensitive and can become subfertile after daily use. In most cases the utilization of AI can improve the reproductive efficiency and capacity of such a stallion. The use of low-dose insemination techniques further enhances the number of mares that can be bred with one ejaculate, which again can improve the pregnancy rates for some of the subfertile stallions. More mares can be bred to the same stallion on a given day. The ejaculate can be divided into several insemination doses, permitting a more efficient use of the stallion semen. The number of mares that a stallion can impregnate during the breeding season can, because of this, be increased several folds. The availability of the stallion semen is likely to be increased in those breed associations that allow the use of artificial insemination. Another benefit is that mares that are

abnormal or could not be bred by natural service can still be bred using AI, for example if they have had any physical injuries earlier. (AMANN, et al., 2002, BRINSKO, et al., 2011)

The older and more valuable stallions can be used more effectively. Since aging causes degenerative changes in the testes leading to a reduced total number of spermatozoa. Another factor is that spermatozoa from older stallions often have a greater number of abnormalities and poorer livability, reducing fertility further. If you follow a properly spaced semen-collection schedule, reliable semen-evaluation procedures and properly timed inseminations, you can obtain more pregnancies with an older stallion using AI rather than natural service. The mares can be bred at the most opportune time for a maximum chance of conception. When numerous mares are booked for the same stallion, the only realistic approach is the use of AI. It is recommended to collect the stallion every other day. The mares should be inseminated after ultrasonography and/or rectal palpation has detected that ovulation is imminent. Progeny testing is possible using AI. Several conditions are assumed to be hereditary without yet being proved. The genetic pool is increased, since mares that are thousands of miles away from the stallion can be impregnated. This also greatly reduces the shipping costs of mares and stallions. The pregnancy rates are higher using AI than using natural service. (AMANN, et al., 2002; BRINSKO, et al., 2011)

2.5 Disadvantages of artificial insemination

There are, however, some drawbacks when it comes to using artificial insemination in horse breeding, and these are necessary to be aware of. A heightened knowledge and skill is required in order to be successful in AI programs. The ejaculated sperm is very susceptible to injury from the environment. In cases of improper semen collection, handling, processing and insemination technique, the pregnancy rates can be lower. The equipment and supplies needed for AI can increase the cost of the AI program. On the other hand, the expenses are generally decreased on a per mare basis, since multiple inseminations can be performed from one ejaculate. The risk of human injury is increased when using an artificial vagina, so a proper training of the people involved beforehand is essential. Due to the heightening of semen processing and handling requirements, even further training is required to be able to reach a level of competence to achieve satisfactory pregnancy rates using low-dose insemination. The equipment costs for hysteroscopic inseminations are relatively high in comparison to those used in manual insemination practices. (AMANN, et al., 2002, BRINSKO, et al., 2011)

2.5.1 Advantages of cooled shipped semen

There are several advantages when using cooled semen. There are eliminated costs and stress of shipping the mare and/or foal to a breeding farm. The possibility of disease

transmission is reduced since there is no exposure of the mare and foal to a new environment. The genetic pool is increased, since breeders often will use a local stallion rather than incur the cost of shipping the mare in cases of natural breeding programs. The use of genetically inferior stallions will be reduced, thus increasing the genetic merit of the breed. Also, the cash outlay for the mare will be reduced. (AMANN, et al., 2002, BRINSKO, et al., 2011)

2.5.2 Disadvantages of cooled shipped semen

There are some disadvantages coming with cooled shipped semen too, including considerable costs involved in the collection, packaging and shipping of the semen to the owner of the mare. Increased veterinary costs are involved in the examination of the mare as well as the hormonal control of her cycle. In order to properly collect, evaluate, extend and prepare the semen for shipment, considerable technical skill and equipment are required. The semen of all stallions are not always suitable for shipment, and the personnel receiving the semen must have the required skills necessary to handle the semen appropriately prior to and during insemination. It is often necessary to follow up the reproductive management, including teasing, ultrasound and breeding in relation to ovulation for maximum reproductive efficiency, and this can in many cases be insufficient. To sum up, the advantages with cooled transported semen usually outweigh the disadvantages, and the main result will be improved quality of the offspring. (AMANN, et al., 2002, BRINSKO, et al., 2011)

2.5.3 Advantages of frozen semen

During recent years the use and acceptance of frozen semen has increased significantly. Certainly, there are many advantages of using frozen-thawed semen. It is cheaper to transport liquid nitrogen containers instead of a horse, and the mare can stay at the farm. There have been tragedies of mares having foals die at the breeding farm or of the mares getting exposed to various diseases. Even if the stallion is ill or performing at events, the breeding season can still go on. Also, the differences between the breeding seasons in different parts of the world, like the northern and southern hemispheres, will not be a problem. The semen of very valuable stallions can be stored and used later, and their genetics can be carried on further even after their death. Semen can be stored “indefinitely”, and the scheduling of breeding has much greater flexibility. The stallions can be selected regardless of their geographical location, so the genetic pool and the quality of the breed can be improved. There is a so-called worldwide access to superior genetics. A young stallion can be gelded after having his semen frozen and stored. Having the semen available at the breeding farm can reduce the cost of breeding a mare, and the stallion can be used in other activities during the breeding season. (AMANN, et al., 2002, BRINSKO, et al., 2011, KUBIAK, 2013)

2.5.4 Disadvantages of frozen semen

However, there are also some disadvantages associated with using frozen equine semen that will have to be considered. The processing, packaging, freezing, and insemination of the semen require considerable technology and skills. Therefore, it is important to send the stallion to a center specializing in freezing of semen with expertise and skilled personnel. Considerable costs and intensive management is needed to prepare the mare for insemination with frozen semen for a maximal fertility. Some breed association requirements will not permit the use of frozen semen, or there will be extra paperwork or costs in association with it. Not all stallions will freeze semen (~20%). There are limitations on how many doses can be obtained from a given stallion, the maximum number of insemination doses from one stallion a year is likely to be no more than 300 to 400. Most mares will be bred several times during each cycle, so a stallion can provide about 100 to 200 mares to be bred a year. A reduction in the genetic pool is a possibility if the mare owners have access to the semen of select sires. A reduced pregnancy rate is the most serious disadvantage with frozen semen, due to slightly lower fertility, but there is room for improvement in this field as the interest and distribution increases. (AMANN, et al., 2002, BRINSKO, et al., 2011, KUBIAK, 2013)

2.6 The distribution of the techniques

2.6.1 The use of frozen equine semen

A scientific article released in 2009 underlines the great differences in the advances of frozen thawed semen in the cattle industry compared to the horse industry. "For the last 30-40 years the dairy cattle industry has selected the bulls based on their ability to produce sperm that withstand the stresses of standard freezing protocols." (BARBACINI, 2009) For horses, on the other hand, the situation is completely different: "On the contrary, the horse industry has never applied such a selection to breeding stallions and this resulted in a wide variation in semen freezability among individuals. In fact, many scientific reports published over the last 20 years showed that, based on their post-thaw motility, 20-40% of the stallion population produce spermatozoa that do not withstand the cryopreservation process." (BARBACINI, 2009) In an attempt to overcome this problem, the so called 'test freeze' technique is being used, and it has given great results in order to increase the percentage of 'freezable' stallions. Stallions showing poor semen quality were excluded for the commercial semen cryopreservation. The export of frozen equine semen is rather recent compared to the bovine industry, therefore the standardization of rules is lacking for some countries regarding the equine industry. "Regulations vary dramatically between countries, from requiring only a permit for importation to several months of quarantine of the stallion or semen. There is a

lack of standardization with regard to requirements, testing, interpretation of results, and means of prevention of the disease.”(METCALF, 2001) The reason for this may be that the bovine industry is much more strictly regulated by the federal attention because of food animal industry. For this reason the equine semen transmission is not as strictly regulated as the bovine industry is. “Disease vectors that have the potential to spread to equids through transported semen – bacteria, viruses, yeast and fungi – either through blood-borne or direct contamination, will always exist.” (METCALF, 2001) It is therefore very important with standardization of the testing procedures, to agree on the interpretation of results, and those who prepare and receive the semen must have conscientious vigilance. Transmission of known diseases must be prevented as an important aid in controlling the transmissible agents in equine semen. (BARBACINI, 2009, BRINSKO, et al., 2011, METCALF, 2001, SQUIRES, et al., 2003)

3 Summary

I feel like I have learned a lot about the use of AI in horses, why it has become more and more important and widely used. It is much easier to use one stallion to breed several mares, and distance is no longer that big of an issue. Less semen is needed and much less work per insemination and higher sperm concentration. When doing AI, examination of the semen quality is easily done, and the stallion can be evaluated quickly. I have got to see and participate a lot in the insemination procedure, all the way from stallion handling and semen collection to the insemination of the mare. This way I have learned more of the practical aspect of breeding, like why it is important to train the stallion to mount the phantom, how to prepare the AV correctly, storing of the cooled semen and how to inseminate in a proper way.

I have compared the different methods and practical aspects of artificial insemination in horses using cooled shipped and frozen semen. Certainly, there are both drawbacks and benefits in both methods. In my opinion, the more experience we get in these areas, the more efficient breeding can take place. I think it is important to face the challenges and improve the areas that do not work at the moment. Especially regarding the frozen semen we still have much more investigation to do. We do have much information available, but the more knowledge we gain in these fields, the more I realize there is still to learn. I hope we can use the information we have and expand the horizons in this industry even more in the future.

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