

Manual of Methods for Preservation of Valuable Equine Genetics in Live Animals and Post-Mortem



Kindra Rader, Charles C. Love, Charlene R. Couch and Katrin Hinrichs

Manual of Methods for Preservation of Valuable Equine Genetics in Live Animals and Post-Mortem

Kindra Rader^a, Charles C. Love^a, Charlene R. Couch^b
and Katrin Hinrichs^a

^a College of Veterinary Medicine & Biomedical Sciences
Texas A&M University, College Station, TX and

^b The Livestock Conservancy, Pittsboro, NC

Published by **The Livestock Conservancy**

P.O. Box 477, Pittsboro, NC, 27312

www.LivestockConservancy.org

©2018

Contents

Introduction to genetic preservation	1
I. SUMMARY OF METHODS FOR PRESERVATION OF GENETICS FROM LIVE ANIMALS	5
A. Tissue collection for cloning and the basics of cloning	7
General Considerations	7
Taking a Tissue Sample	7
Culture and freezing of cells	8
B. Semen collection and freezing	10
At a breeding center	10
How do I choose a semen collection and freezing laboratory?	10
My stallion has never bred before. What are the typical procedures that he will go through at the collection center?	11
How long will the stallion stay at the breeding center?	11
How many insemination doses of semen will I get?	11
Will having had semen collected change my stallion's behavior?	12
How much will it cost?	12
At the farm.....	13
Inexperienced stallions or stallions reluctant to ejaculate.....	13
Phantom.....	13
Mount mare	13
The artificial vagina	13
Use of mount mare and condom.....	13
Use of artificial vagina on ground	13
Manual technique.....	14
Pharmacological induction of ejaculation	14
Incapacitated stallions, and when it is not possible to collect an ejaculate -- What if my stallion will not ejaculate with any of the above?	14
Consult a behaviorist or veterinarian experienced in stallion reproduction.	14
Electroejaculation	14

Epididymal aspirate.....	15
Testicular biopsy	15
When you do collect semen, how do you send the semen to the freezing laboratory? .	15
Frozen semen doses and ICSI	16
Considerations	16
ICSI doses and cuts, and re-freezing of previously-frozen straws	16
C. Embryo recovery, transfer, biopsy, cryopreservation.....	18
Overview of embryo transfer	18
Performing embryo recovery and transfer	19
Embryo biopsy	19
Embryo cryopreservation (freezing or vitrification).....	20
D. Oocyte recovery, handling and cryopreservation	22
Overview of oocyte recovery	22
Transvaginal ultrasound-guided follicle aspiration (TVA)	23
Follicle aspiration through the flank.....	23
Scheduling for oocyte recovery: immature vs. mature follicles.....	24
Recovery from immature (small) follicles.....	24
Oocyte recovery from the stimulated dominant follicle	24
Oocyte vitrification	25
E. Intracytoplasmic Sperm Injection (ICSI)	26
Application of ICSI.....	26
F. Oocyte transfer to the oviduct	28
G. Intrafollicular oocyte transfer.....	29
II. SUMMARY OF METHODS AND PROTOCOLS FOR PRESERVATION OF GENETICS FROM ANIMALS POST-MORTEM	31
Outline of available methods for genetic preservation post- mortem.....	33
A. Recovery and handling of somatic cell tissue for future cloning	37
General considerations.....	37
Protocol	38
Expected results.....	39

B.	Removal, handling and shipment of testes	40
	General considerations.....	40
	Protocol	41
	Expected results.....	43
C.	Emergency freezing methods for somatic tissue and sperm	44
	Freezing medium components.....	44
	Protocol for somatic tissue.....	45
	Protocol for sperm.....	45
D.	Cryopreservation of testicular tissue from the testes.....	46
E.	Removal, handling and shipment of ovaries	47
	General considerations.....	47
	Protocol for ovary removal.....	48
	Protocol for ovary handling and shipment.....	50
	Expected results.....	51
F.	Cryopreservation of ovarian tissue from the ovaries.....	52
G.	Recovery of oocytes from harvested ovaries	53
	General considerations.....	53
	Protocol for oocyte recovery.....	53
	Protocol for shipment of isolated oocytes	58
	Expected results.....	58
H.	Intrafollicular transfer of isolated oocytes	60
	General considerations:.....	60
	Procedures:.....	61
	Laboratory preparation.....	61
	Mare-side preparation.....	63
	Flank approach:.....	63
	TVA approach:.....	64
	Laparotomy approach:.....	65
	Expected results.....	66
	Some Equine Assisted Reproductive Technology Resources	67

Introduction to genetic preservation

Advances in equine reproductive technologies make it possible for the individual horse owner or breed association to preserve priceless genetics—even after the animal’s life has ended. These samples of tissue, or eggs or sperm, may represent the last remaining genetic information from a valuable horse, or lineage, or even an entire breed.

This manual is meant to be used as a quick guide to the Assisted Reproductive Technologies (ARTs) that are currently available to preserve important genetic material in living horses (**Section I**, starting on page 5), or in a worst case scenario, after a horse’s death (**Section II**, starting on page 31). Procedures to follow on the farm to secure samples in the case a horse is dying or has just died are presented in detail, in the case that this manual is the only source of information available at the time.

Individual owners and breeders can use these techniques as needed to secure samples for preservation of valuable genetics. When these techniques are used by breed associations to preserve breed genetics for long term use, or as a “backup,” in case of the catastrophic loss of breed representatives, breed associations should carefully select the animals from which samples will be preserved, based on both genetics and economics. This optimally will be a broad representation of the breed, including both major and minor bloodlines. The collection may reflect the genetic ratios in place at the time the tissues are banked, but preferably will contain equal representation of all genotypes present in the breed, whether currently desirable or undesirable.

For breeds with populations in more than one country, or for owners who wish to ship genetic material to other countries, it is worthwhile to investigate health testing requirements in all possible countries to which germplasm (gametes) or tissue samples might be exported. Following required procedures such as quarantine and testing before collection and storage of tissue samples, including use of approved quarantine facilities, may add to the cost of collecting the samples, but will preserve options for future transfer of critical genetics between countries.

Genetic preservation refers to saving samples of DNA from your animal. **DNA** (deoxyribonucleic acid) is the genetic code that serves as the “blueprint” for making your animal the way it is. Just as these sentences are composed of letters, DNA is made by stringing together “letters” (nucleotides); however in the case of DNA there are only 4 letters to choose from. Each animal’s DNA incorporates about 3 billion of these letters, however, and thus there is a huge potential for variation in the way the letters are ordered. Unless your animal is an identical twin, a clone, or an animal inbred to the point where there is no genetic variability (such as a lab mouse) the sequence of your animal’s DNA is unique.

Each animal’s DNA codes for about 30,000 different proteins, and many times that number of signaling molecules that govern how the DNA is used. Many of the proteins produced from “decoding” the DNA are enzymes that serve to regulate how other molecules, such as carbohydrates and fats, are formed. The amount and the character of the proteins produced from the DNA, and the unique combination of these in your animal, along with which cells use which sections of the DNA, all dictate how your animal develops and what traits it has.

Since the amount of DNA in a cell is huge, the DNA is divided and packaged into separate **chromosomes**. For example, horses have 64 chromosomes in each cell, and donkeys have 62 chromosomes in each cell. Each chromosome is a piece of DNA, wrapped around and attached to proteins and other molecules that help to organize it and also help govern which segments (genes) of the DNA will be used by that cell and which are not used. The chromosomes are organized within a separate compartment of the cell, the **nucleus**.

While cells of different tissues use different sets of genes in the DNA, the nucleus of every cell in a given animal has a copy of ALL the DNA. Thus, each cell has exactly the same DNA makeup. The exceptions to this are the gametes, the **eggs** (oocytes) and **sperm**. Body cells (somatic cells) have two copies of every chromosome, one the animal got from its dam and one from its sire. For example, horses have 32 different kinds of chromosomes; two of each kind gives them the total chromosome number of 64. The gametes, however, are prepared to make the next generation. In the gametes, the number of chromosomes has been reduced to only one of each kind of chromosome, so that when the oocyte and sperm come together in fertilization, the resulting embryo has again the normal complement of two chromosomes of each kind.

Genetic preservation can be divided into three main areas

1. **Preservation of somatic cells.** This involves saving cells from your animal's tissue, typically from under the skin of your animal. The complete chromosome makeup in the nucleus of the cells can be used via cloning (somatic cell nuclear transfer) to produce a foal with exactly the same DNA as your animal. While theoretically an animal could be reproduced by only knowing the order of the "letters" (the nucleotide sequence) of the DNA – think *Jurassic Park* – currently cloning requires the **nucleus of a living cell**. This is because all of the proteins, signaling molecules, and other messages accompanying the DNA in the nucleus are important to allow the DNA to be used normally.
2. **Preservation of gametes.** This involves saving unfertilized oocytes (eggs) or sperm from your animal. These gametes can then be used for fertilization to produce offspring from your animal. The eggs and sperm must be kept viable, that is, able to perform all the functions needed for fertilization and support of embryo development.

Sperm can be collected from your stallion via ejaculation, or can be collected from the testicles and attached tubules (epididymis) at the time of castration, or after death.

Oocytes can be repeatedly collected from the ovaries of live mares, with minimal chance of injury, or can be collected from the ovaries after the mare's death.

3. **Preservation of embryos.** Embryos can either be recovered from the uterus of mares after breeding, or can be produced *in vitro* (in the laboratory) from oocytes and sperm, using methods for *in vitro* fertilization (such as intracytoplasmic sperm injection, ICSI) and culture of the fertilized egg in an incubator. Embryos from either source maintain viability well after cryopreservation (freezing), take up little space, and can be transferred to a mare after thawing, to immediately produce a pregnancy that can lead to a live foal.

**I. SUMMARY OF METHODS FOR PRESERVATION OF
GENETICS FROM LIVE ANIMALS**

A. TISSUE COLLECTION FOR CLONING AND THE BASICS OF CLONING

General Considerations

Cloning is a way to produce an embryo that has exactly the same DNA makeup as the *donor* animal. The cloned embryo can be transferred to a recipient mare, which will carry the foal to term and serve as its dam.

Cloning is performed using a “host” oocyte that is ready to be fertilized. In normal fertilization, the sperm would join with the oocyte to fertilize it, with the sperm contributing half the DNA and the oocyte contributing half the DNA to form the new embryo. In cloning, also known as somatic cell nuclear transfer, we remove the oocyte’s DNA, and instead of a sperm, we insert a regular cell (somatic cell) from the donor animal into the oocyte. This cell contains the DNA of the donor animal in its nucleus, so now the oocyte has the full complement of chromosomes from the donor animal. The oocyte is stimulated to divide and develop into an embryo, which in about 7 days can be transferred to a recipient mare. The resulting embryo has the DNA of the donor animal. The DNA directs the resulting embryo to have the attributes of the donor animal – at least those attributes that are determined by genetics!

Cloning is a useful means of preserving specific desirable genetic lines. Whole populations can be rejuvenated by cloning animals that are no longer in the gene pool, but whose genetic material was saved in foresight. Cloned animals can be used for breeding and appear to have normal fertility and completely healthy offspring. This is especially valuable if the original animal was lost or was gelded before his or her genetic value was known. Although the cost of cloning an equid is high (about \$70,000 commercially at the time of writing), prices continue to decrease.

While producing a cloned embryo may be expensive, the cost of tissue harvesting (which entails removing a pea-sized bit of tissue from under the skin of the donor animal) and freezing of the somatic cells grown from that tissue, are affordable. Once the cells are frozen, the decision to clone the animal can be made in the future. The cost for collection of the tissue is variable (this depends upon your veterinarian, who will take the tissue sample); processing of the sample for cell culture and storage is about \$1000-\$2000 at the ART or cell culture laboratory.

Taking a Tissue Sample

Taking tissue samples from a domestic equid is a simple process. Ideally this should be done while the animal is alive and healthy, instead of as a last minute rescue attempt on a sick or dying animal. This will ensure that the tissue harvested is healthy and viable, and also will avoid stressful time limitations that may jeopardize the success of the process.

Your veterinarian can make a visit to your location and take subcutaneous tissue samples from the neck, chest, or gum of the animal. Ideally samples should be collected from locations that are not regularly exposed to the sun, will heal quickly, and will not be overly noticeable during healing, especially if this is a show animal and cosmetic appearance is of importance.

The veterinarian will need specific supplies and media, including sterile gloves, disinfecting materials for the sampling site, scalpel and forceps. These, and detailed instructions for taking, packing, and shipping the samples, are typically provided by the ART laboratory to which the tissue will be sent. The animal will need to be sedated during the procedure, and you will want to do the procedure in a quiet, low-dust environment if possible, preferably inside and out of the sun. For samples taken from skin, a small area beneath the mane or between the forelegs will need to be shaved and scrubbed as if for surgery. Avoid the area around the tail, as this has a high amount of fat cells, which do not grow well in culture. The veterinarian will block the area with a local anesthetic, and make a small skin incision using a scalpel blade. Tissue from under the skin is obtained, and the site is closed with a few sutures and should heal quickly. Horse owners will need to watch the site to make sure it stays clean of debris and dirt to prevent any infection.

Ideally, if an animal is aged or is facing a life-threatening illness or injury, it is wisest to collect tissue samples from two or more locations. This is a safeguard in the case one sample is contaminated or does not grow well in the laboratory.

Recently it has been indicated that cells obtained from bone marrow (mesenchymal stem cells) may work better for cloning horses than do skin or gum cells. Your veterinarian may take a bone-marrow aspirate (typically obtained from the breastbone while the horse is standing, tranquilized).

Culture and freezing of cells

Once the tissue samples reach the laboratory, they are cut into very small pieces and cultured in a growth medium in an incubator. Under these conditions, the cells of the tissue multiply and typically in a week to 10 days, cells can be seen growing out of the tissue onto the surface of the dish.

Bone marrow aspirates are treated to try to isolate the stem cells from the aspirates, and then these cells are cultured in a special medium.

Culture is typically performed for three weeks or more, until a large number of cells is obtained. The cells are then frozen in straws and can be stored indefinitely in liquid nitrogen for later use for cloning. The owner will need to set up long-term safe, monitored storage with a reputable storage facility for the frozen cells.

Almost all tissue obtained from living animals will grow cells in the laboratory, unless there has been contamination of the sample with bacteria, fungus or toxins during the biopsy procedure, or exposure to heat, sunlight or other sources of radiation. The cells freeze well, and have good viability after freezing. However, the ability of the cells to produce a viable cloned embryo is not known until the cloning procedure is attempted. In our laboratory, 1 of 12 cell lines with which we have worked did not provide viable embryos.

B. SEMEN COLLECTION AND FREEZING

It is difficult to predict when a valuable breeding animal might leave the gene pool, which makes collection and preservation of genetic material from outstanding individuals during their prime of utmost importance. Frozen semen can expand the breeding career of most stallions, extending their normal book limits within a season, allowing foal crops to be born in multiple countries, and allowing foals to be born after the stallion's death. However, it is important to note any breed registry rules or restrictions regarding the use of frozen semen from stallions. These rules can differ if the semen is used prior to or after the stallion's death.

If you normally use pasture- or hand-breeding methods in your herd, it is wise to consider having your stallion trained to mount and have semen collected using a phantom (dummy mare) to ensure that semen can be frozen and stored from him. This will also allow you to have the semen analyzed, allowing you to make better management decisions regarding your pasture-breeding program to potentially increase success.

When scheduling semen collection for freezing purposes, one must consider the current demands on the stallion. Is he still being used in active competition? Is he in high demand as a breeding sire? Will he be travelling internationally? All of these factors may influence the collection schedule.

If a stallion has a large book of mares during the breeding season, scheduling semen freezing collection days in the off-season may be most feasible. However, breeding managers and stallion owners may be wise to consider freezing any "extra" breeding doses collected from that animal during the breeding season, that were not shipped for various reasons. Perhaps the intended mare that day ovulated and you were left with an unused breeding dose. Instead of discarding that semen with the mindset of "We will get her next time," if the resources are available (a local practice freezes semen, or semen can be shipped to an ART laboratory), that dose should be frozen. If the stallion were to colic the next day and die, it would be terrible to think back to the one dose you poured down a sink drain instead of putting it into your storage tank.

At a breeding center

How do I choose a semen collection and freezing laboratory?

While there are many stallion-related factors that contribute to the post-thaw quality of frozen sperm, the quality of the semen is also dependent on the laboratory that processes it. Working with a laboratory that performs the procedure routinely, and that can provide you data on the post-thaw motility and pregnancy rates of semen that they have frozen, is essential to ensure that the best possible semen collection, handling and freezing techniques are used.

My stallion has never bred before. What are the typical procedures that he will go through at the collection center?

If the stallion has never bred before he will be trained to mount a mare or phantom (dummy mare) and to “serve” the artificial vagina. Training should be done by experienced handlers so that the stallion will learn to work in as safe and relaxed a manner possible, to allow for easy, routine semen collection. The length of time required for a stallion to learn to breed varies. Some stallions breed immediately while others require several days or longer as the handlers try different stimuli and situations to encourage breeding behavior.

Once semen is collected, sperm quality will be evaluated to identify those stallions that have extremely poor sperm quality. The ability of a stallion’s sperm to freeze well under different freezing conditions can be tested prior to “banking” a large amount of frozen sperm.

Stallions that have not had ejaculates collected or have not bred mares for a while will have stored sperm (sperm that has stayed in the epididymis for days or weeks) that can be old and of poor quality. These stallions will need to be “cleaned out” by repeated ejaculation to remove the poor-quality sperm so that an accurate representation of his fresh sperm quality can be determined.

How long will the stallion stay at the breeding center?

This will depend on the training time, the cleanout time, and the amount of sperm that the client wishes to freeze. An experienced breeding stallion of normal fertility that has been actively breeding (and so is “cleaned out”) typically has semen collected every other day or three times a week, and could provide 6 to 8 frozen breeding doses per collection day.

How many insemination doses of semen will I get?

A dose is a deceiving concept because it assumes that a “dose” will result in some degree of fertility. While we can identify those stallions with very poor quality sperm that would make a poor freezing candidate, we are less accurate at assuring that “good quality” frozen sperm results in pregnancy. Therefore, the only true test of fertility of frozen semen is to actually breed mares. Nevertheless, we “calculate” a dose based on post-thaw sperm motility, which gives a starting point regarding the number of straws to use per insemination.

Given the above considerations, we can present an idea of what might be a “typical” collection from a mature, fertile stallion that is on an every-other-day collection schedule:

- A. 6 billion sperm in the ejaculate
- B. Sperm is typically frozen with 100 million sperm per straw, thus this ejaculate would give 60 straws

- C. Sperm progressive motility (the proportion of sperm moving in a linear manner, thought to represent sperm that are functional) may be around 35% after thawing
- D. Considering a breeding dose to be 250 million progressively motile sperm, 8 straws would be needed to provide this number of sperm from this semen sample. Thus, this ejaculate would provide 7.5 breeding doses.

Obviously, every stallion is different; the number of sperm in the ejaculate and the post-thaw motility can vary even between ejaculates for the same stallion. The size of the horse also matters. Smaller horses and ponies may have correspondingly smaller testicles and may produce fewer sperm per collection than do large stallions. In addition, different laboratories prepare the breeding doses differently. For example, a laboratory may have a standard breeding dose of 8 straws, and so they vary the concentration of semen within the straws so that for every stallion and ejaculate with which they work, there are a sufficient number of progressively motile sperm contained in this number of straws.

The number of mares that can actually be inseminated with a “breeding dose” varies depending upon the breeding schedule used; some effective frozen-semen breeding protocols call for breeding mares at two different times before ovulation, and thus two doses are needed per cycle.

The pregnancy rate with frozen semen is often less than that when fresh semen is used; less than 50% pregnancy per cycle is typical.

Will having had semen collected change my stallion’s behavior?

This depends upon the stallion. Some stallions can handle the experience without any change in non-breeding related behavior, while others may become more “stallion-like.”

How much will it cost?

The cost of training (~\$60/hour) and sperm cleanout (~\$150/collection) will vary. At Texas A&M University, we perform a “test freeze” in which several different freezing diluents (extenders) are tested to determine which is best for a particular stallion (~\$1200). If the client decides to continue to collect sperm for freezing and banking, the cost is approximately \$750/ejaculate. Board for a stallion at a breeding center is typically more expensive than for mares or geldings, and can be up to \$35 or more per day. The total cost of collection will depend on the number of doses collected, but is generally in the range of \$2500-3500.

At the farm

Inexperienced stallions or stallions reluctant to ejaculate

Phantom

If a phantom (artificial or dummy mare) is available at the farm, this greatly simplifies training of inexperienced stallions. A live mare (stimulus mare) may be shown to the stallion before or during the time he is mounting the phantom. Many stallions will even mount the phantom with no stimulus mare needed, or with a stimulus mare available that may not be in heat.

For stallions that readily accept mounting a phantom, this is a convenient method to collect semen and tends to be safer since a live mare is not being mounted and thus is not able to harm him or the personnel.

Mount mare

Some inexperienced stallions, and some stallions that have bred by natural means (live mare), are not interested in the phantom and therefore require the use of a live mare to mount. In addition, some smaller horses and ponies cannot successfully mount a horse-sized phantom and require a pony-sized mount mare. Additionally, if no phantom is available on the farm, a live mare is required as a mount mare. The mare must be in estrus, or can be a mare whose ovaries have been surgically removed, with or without estrogen treatment.

The artificial vagina

Once the stallion is mounted on the phantom or mount mare, a separate person (not the handler) approaches, holding the artificial vagina (AV), and diverts the stallion's penis into the artificial vagina for collection of semen. If the stallion does not ejaculate in the AV, changes can be made to the temperature and pressure of the AV or to the handling of the penis and AV. Most stallions can have ejaculates collected using an AV.

Use of mount mare and condom

Some stallions may be difficult to collect with an AV. In most cases this is due to improper preparation of the AV. This can also be seen in some stallions that have bred by natural cover their entire breeding career. Use of a stallion condom on the penis while the stallion is covering a mare can be attempted, but many stallions do not like this either. It has the additional disadvantage that the semen is contaminated by contact with the penis. It is an undesirable method of semen collection but can be used if there are no other options.

Use of artificial vagina on ground

Stallions with musculoskeletal pain or physical limitations can have semen collected while they are standing with four feet on the ground by applying the AV to the penis after erection has been achieved. Some stallions respond well to this technique; others are more hesitant.

Manual technique

Semen can also be collected from the standing stallion in some cases by applying a condom to the penis or placing a plastic bag over the penis, and applying warm compresses and pressure manually to the base and glans of the penis. This may allow semen to be collected when no AV is available.

Pharmacological induction of ejaculation

In situations in which standard semen collection is not possible, pharmacological (chemical) induction of ejaculation can sometimes be used to collect sperm for cryopreservation. In this way, the stallion can have semen collected at home if necessary, under the supervision of a veterinarian experienced in this technique. A medication is administered to reduce the threshold for ejaculation, which sedates the horse. The veterinarian may have to try different doses and combinations of medications over a series of days to achieve the correct response. If effective, the sedation medication will cause ejaculation. The ejaculate is collected in a bag that was placed over the penis before administering the medication. The collected sperm can be shipped directly to a cryopreservation facility; however, it is best if the stallion is located at the site where cryopreservation occurs to maximize the quality of the semen collection and the processing. Cost is variable for the sedation procedure (your veterinarian); cryopreservation of shipped semen can cost about \$500-\$750 per ejaculate.

Incapacitated stallions, and when it is not possible to collect an ejaculate -- What if my stallion will not ejaculate with any of the above?

Consult a behaviorist or veterinarian experienced in stallion reproduction.

Stallions may not ejaculate for a variety of reasons, ranging from physical limitations causing pain, to poor AV preparation and handling. If a stallion does not ejaculate during the first attempts at collection, many methods can be used to help the stallion to ejaculate. While you can attempt to “solve” these problems at home, this has the potential to result in behavioral problems and exacerbation of the underlying problem. Therefore, consultation and referral to a veterinary specialist in stallion reproduction is the best alternative.

Some of the additional methods that the specialist might use to get a stallion to ejaculate include using different stimulus mares, mares in better estrus, warm compresses, changing the temperature and pressure of the artificial vagina, combining the artificial vagina with pharmacological stimulation, housing the stallion differently, and putting the stallion out with estrous mares for a while to get experience, among other variations.

Electroejaculation

Electroejaculation is performed by placing a probe into the rectum and applying electrical pulses, which stimulate the reproductive tract and can cause the animal to ejaculate some

semen. This method is used commonly in bulls, with the bull standing in a chute. However, stallions are sensitive to the procedure and it must be done with the stallion under general anesthesia. The procedure carries the risk of perforating the rectum, which can result in death. Additionally, electroejaculation in the horse is often unsuccessful, so it should be considered as a very last option. If the stallion is recumbent and expected to die, removing the testes and epididymides after death (see below) and freezing the recovered sperm is probably the better approach.

Epididymal aspirate

If an ejaculate cannot be obtained from a stallion, and the stallion is not near death and the owner does not want to castrate him, sperm (enough to be used for ICSI) can potentially be obtained by aspirating the tail of the epididymis with a needle and syringe in the live stallion. While sperm may be obtained, the needle puncture can result in sperm leaking into the tissue of the epididymis, which can cause formation of granulomas (non-bacterial abscesses) and may block the epididymis so that ejaculation of sperm is not possible in the future. This again is a last-ditch method, but it has been used in humans that have no ability to ejaculate, in order to recover sperm for ICSI.

Testicular biopsy

If an ejaculate cannot be obtained, and there are few or no sperm in the epididymis, then a testicular biopsy may provide sperm for ICSI. This again is a last-ditch method, but it has been used for humans when the man produces no ejaculate, or an ejaculate with no sperm in it.

When you do collect semen, how do you send the semen to the freezing laboratory?

If at all possible, the stallion should have semen collected at the location where the semen will be frozen. This will guarantee that the sperm is handled in the best manner possible and will increase the quality of the resulting frozen semen. Semen freezing facilities cannot be responsible for the quality of frozen sperm obtained from semen collected in the field and shipped to the center.

However, if a stallion is in an emergency situation (dying) or if there is no semen freezing facility to which the stallion can be shipped, the semen can be collected on the farm and shipped to the freezing facility. To do this, the semen is extended to about 25 million sperm per mL in a standard insemination extender, and sent in a cooled semen shipment container, such as an Equitainer, by overnight courier.

Frozen semen doses and ICSI

Considerations

For a description of ICSI (intracytoplasmic sperm injection) see **Intracytoplasmic Sperm Injection (ICSI)**, page 26.

Many stallion owners do not consider banking large amounts of frozen semen from their stallions until their offspring are proven and the demand for their stallion has increased enough to justify the storage costs. Unfortunately, there is a risk that a stallion may die before his first foal crop is proven, and thus perhaps only one “test” ejaculate has been frozen, or only epididymal sperm frozen post-mortem is available.

In other cases, stallions become aged as their reputation as a sire reaches a pinnacle. Demand for their semen goes up, but this occurs at a time when their semen quality is decreasing. Freezing semen in a stallion’s golden years may present new challenges as the stallion’s fertility level changes. If semen is exceptionally poor when frozen and thawed, many straws may be necessary to inseminate a mare in the hopes of achieving a pregnancy. This uses stores of frozen semen quickly.

ICSI doses and cuts, and re-freezing of previously-frozen straws

Freezing of one ejaculate of semen in “ICSI doses” should be considered for every genetically valuable stallion.

Frozen semen is typically used in standard insemination doses of from 4 to 10 straws, each with 100 million sperm, to inseminate a mare. The pregnancy rate after insemination with frozen semen varies but is often less than 50%. Thus, only a limited number of foals can be produced by standard insemination with the semen frozen from one ejaculate. For this reason, freezing semen for standard insemination can become an expensive procedure in order to collect enough breeding doses to help assure that the desired quantity of foals are produced.

In contrast, freezing ICSI doses of about 1 million sperm per straw will allow the production of very large numbers of semen straws from one ejaculate that can be used for ICSI procedures (typically about 400 straws from one ejaculate)! In many cases, the sperm is of good enough quality that only a portion (cut section) of a straw provides enough viable sperm for an ICSI attempt, thus extending even further the number of fertilization procedures that can be performed. This gives the promise of extended foal crop production from a stallion after he becomes infertile due to age, accident or disease, or dies.

If a stallion is dead or is no longer producing viable sperm, and ICSI doses were not frozen from him previously, a standard-dose frozen semen straw (typically containing 100 million sperm) can be thawed, diluted, and re-frozen into ICSI doses of 0.5 to 1 million sperm each, depending

on the original post-thaw motility. This can result in approximately 30 to 200 ICSI-dose straws from the one standard frozen semen straw. This greatly increases the potential to produce foals from the given amount of frozen semen from that stallion.

Again, if the refrozen semen has good post-thaw motility, it may be possible to use cuts of this refrozen ICSI-dose straw. Each ICSI-dose straw may then yield 4 to 10 uses, depending on the size of the cuts needed to provide the semen necessary to complete an ICSI session.

Limited semen stores can further be extended for ICSI by grouping oocytes from mares to be fertilized with that stallion's semen. Veterinarians can coordinate mare follicle aspirations to be performed on the same day, so that oocytes from multiple mares are sent to the ICSI laboratory for fertilization with the same stallion on the same day. This means that only one ICSI-dose straw or ICSI cut needs to be thawed to fertilize oocytes from multiple mares.

It is important to remember that once semen has been processed into ICSI doses or cuts, it is no longer useful for traditional frozen semen breeding methods. Separate inventories will need to be kept, and contract wording may need to be made more specific, with stud fees reflective of the additional expenses and risks taken by mare owners attempting ICSI for the purpose of breeding to a stallion.

Additional Note: Some breed registries have placed restrictions on the use of frozen semen after the death of a stallion. Stallion owners should consult with their breed registry rules prior to sending their frozen semen straws for reconstitution.

C. EMBRYO RECOVERY, TRANSFER, BIOPSY, CRYOPRESERVATION

Overview of embryo transfer

Embryo recovery and transfer has become commonplace in most equine industries. In this procedure, the valuable mare is inseminated, and the embryo is flushed from her uterus about seven days after she ovulates. The recovered embryo is then transferred to the uterus of a recipient mare, which carries the desired foal to term. Embryo recovery and transfer is a multi-step process involving careful management of the donor mare's reproductive cycle, as well as coordination and synchronization of the reproductive cycle of a recipient mare that is designated to carry the foal.

This practice benefits mare owners who wish to keep their mares in active competition or wish to produce more than one foal per mare per year. Embryo recovery and transfer can be performed as often as once every 2 weeks or so, throughout the breeding season (the cycle between heat periods is shortened by use of hormones). This procedure also avoids the potential complications of pregnancy and foaling for an extremely valuable mare, and allows foals to be produced from a mare that has a limited ability to carry her own foal due to aging, repeated pregnancy loss, or other limitations.

Embryo recovery requires that the mare is able to conceive and to carry the resulting embryo for the first ~7 days of development. Mares that have ongoing fertility issues that prevent conception or hinder the early development of an embryo, including recurring anovulatory follicles, post-breeding endometritis that does not respond to treatment, or cervical and uterine damage from dystocia or other reproductive injury, are generally not good candidates for embryo transfer. However, these mares may be candidates for oocyte recovery and ICSI (see **Intracytoplasmic Sperm Injection (ICSI)**, page 26).

Mare owners may use embryo transfer as a means to preserve embryos from their mares through cryopreservation (freezing or **vitrification**) of the collected embryos. This can be used as a management technique when embryos are recovered late in the year. The embryos can be vitrified and stored, and then transferred the following spring, resulting in a foal born early the following year.

Additionally, embryo recovery can be used with mares that carry genetic diseases or are being bred to stallions carrying a genetic disease. In this case, the recovered embryos can be **biopsied** and the cells used for genetic testing prior to transfer of the embryo. In this way, mares and stallions that carry genetic diseases can be used for breeding and, through selection and

transfer only of embryos that do not carry the disease, still produce completely normal offspring.

Performing embryo recovery and transfer

Embryo recovery is performed with the donor mare standing in stocks – tranquilization is usually not needed. Fluid is introduced through the mare’s cervix into the uterus, and then drained out of the uterus through a filter that will catch the microscopic embryo. Embryo recovery is typically done between 6 to 8 days after ovulation.

If an embryo is recovered, it is then transferred non-surgically (through the cervix) into the uterus of the recipient mare. This can be done either at the same location as the donor mare, or the embryo can be shipped to be transferred on another farm that manages recipient mares.

The success rate of embryo recovery is dependent on many factors, including the quality of management of the donor and recipient mares; the age, health, and condition of the donor mare at the time of breeding; and the quality of semen used, to name a few. In completely normal mares, a typical result is that an embryo is recovered on about 75% of cycle attempts.

The pregnancy rate after transfer of normal embryos to the recipient mare is typically around 75%, but this depends on the quality of the embryo, the skills of the person performing the transfer and the quality of recipient mares used. The cost involved in transferring an embryo to a recipient mare (typically incorporated into the purchase of the pregnant recipient) is often ~\$2000-\$5000.

While many breed associations allow unlimited use of embryo transfer, it is important that each mare owner familiarize themselves with their breed registry’s requirements before utilizing embryo transfer as a means to obtain foals from their mares.

Embryo biopsy

Many genetic diseases have been identified in horses. Some are specific to certain breeds or genetic lines. Some breed associations require diligent genetic testing of all foals prior to registration, in order to determine their status as carriers of these diseases and ultimately help breeders make more informed decisions. However, this kind of genetic testing is performed after all the hard work has been put in to obtain that foal from the desired parents. It is immensely disappointing and costly to owners to find out after birth that a foal is affected by an undesirable genetic disease.

Mare and stallion owners wishing to breed together two animals known to be carriers of recessive diseases, or exhibiting a dominant genetic disease, should exercise caution as the resulting foal may carry, or be affected by, the disease. In such a case, it is possible to recover

embryos from this breeding pair and test the genetics of the embryo to determine the genetic makeup of the potential foal prior to transferring the embryo. In this way, only foals having the desired normal genetic makeup are born.

Currently, embryo biopsy is only performed by specialized ART laboratories. After the embryo is recovered from the donor mare as outlined above (preferably collected on Day 6.5 after ovulation, as the biopsy is easier to perform on a smaller embryo), the embryo may be shipped by air or overnight courier to the ART laboratory for biopsy. The biopsy is performed by taking a small sample of cells from the embryo, using a micromanipulator and micropipette. The cells are collected from the area of the embryo that eventually forms the placenta. Approximately 10 to 20 cells are collected. Because the typical embryo at Day 6.5 - 7 contains thousands of cells, there is minimal effect on the embryo.

After the biopsy is performed, the collected cells are submitted for genetic analysis. The embryo may be shipped to an embryo transfer center for immediate transfer into a recipient mare, or may be vitrified (frozen) to be transferred after the genetic analysis results are available (about one week). If the embryo is transferred immediately, and the results come back with undesirable findings, the pregnancy can be terminated.

The genetic analysis is >95% accurate; however, there is a slight possibility (<5%) of a gene being missed or the DNA not being read correctly.

Knowing the genetic makeup of the embryo gives the owner the choice to avoid pregnancies that will result in affected foals (e.g., homozygous for a recessive affected gene or heterozygous for a dominant gene) or even to avoid producing foals carrying one recessive gene for a disease. In addition, the genetic analysis can determine the sex, coat color, and parentage verification markers of the upcoming foal.

Embryos that have been subjected to biopsy and are transferred immediately have a normal pregnancy rate (>80% in our studies). There is no increase in pregnancy loss after transfer of biopsied embryos, and foals born from biopsied embryos are normal. If the owner chooses to freeze or vitrify the embryo to wait for the genetic results, this cryopreservation may decrease pregnancy rate somewhat (~5 to 10%).

Embryo cryopreservation (freezing or vitrification)

Embryo vitrification is a means to cryopreserve (freeze) embryos. Small embryos, such as those produced by ICSI (see **Intracytoplasmic Sperm Injection (ICSI)**, page 26) and embryos collected from mares relatively early (at ~ Day 6.5 after ovulation) can be vitrified for later use without additional manipulation. Larger embryos (blastocysts), such as those recovered from mares on

Day 7 or Day 8 after ovulation, require manipulation before they can be frozen or vitrified successfully. Blastocysts larger than 300 μm in diameter, which are fluid-filled, are collapsed by puncturing their outer layer using a micromanipulator. This technique allows these embryos to be frozen or vitrified successfully. The frozen or vitrified embryos are placed in liquid nitrogen and can be stored indefinitely. Collapsed, vitrified blastocysts reform their shape quickly when warmed, and have resulted in good pregnancy rates (> 70%) after transfer.

Embryo freezing or vitrification and storage in liquid nitrogen make it possible to store embryos essentially indefinitely without affecting their ability to produce a foal. Embryo freezing or vitrification can be used for reproductive management; for example, to obtain earlier foals from mares that, due to age or chronic breeding issues, do not provide embryos until late in breeding season, or from mares that foal late in the breeding season, or are involved in active competition. Embryo freezing or vitrification also allows embryos to be stored if recipient mares are not available or desirable at the time the embryo is produced. The vitrified embryos can be warmed and transferred in the following years, at a time of the embryo owner's choice or when conditions are suitable.

Another use of embryo cryopreservation is for storage of multiple embryos. Following ICSI, multiple embryos are sometimes produced and mare owners are faced with paying additional stud, embryo transfer, and recipient fees they had not planned on. Vitrification of these additional embryos may give the mare owner an opportunity to work with one embryo at a time until an ongoing pregnancy is achieved, and then negotiate with the stallion owner about what to do with any additional embryos. ICSI can be used to obtain embryos from mares year-round, and if this occurs in months when an embryo transfer is undesired, the embryos may be transferred during the next season.

Embryo vitrification is also helpful for breeders wishing to only transfer embryos with known genetic makeup. Embryos may be flushed or produced via ICSI, biopsied for genetic analysis, and then vitrified pending the results of genetic testing. If the results are undesirable, then the owner has the option to discard the embryo and try again, saving themselves the expenses associated with transfer, recipient mare maintenance, and birth of a foal affected by a genetic disease or other undesirable trait.

D. OOCYTE RECOVERY, HANDLING AND CRYOPRESERVATION

Overview of oocyte recovery

Mares that are unable to carry their own foals often become embryo donors through an embryo recovery and transfer program. But what if they are not good candidates for embryo recovery and transfer, due to fertility issues that prevent early embryonic development? In this case, the mare can become a candidate for oocyte recovery and production of an embryo from the recovered oocyte.

For this procedure, oocytes (unfertilized eggs) are recovered from follicles on the mare's ovaries, and the eggs are then fertilized with sperm from the desired stallion. The methods to fertilize a collected oocyte are outlined in the following sections; these include intracytoplasmic sperm injection (ICSI), in which fertilization is performed in the laboratory (*in vitro*); oocyte transfer (OT), in which the mature oocyte is transferred surgically to the oviduct (Fallopian tube) of an inseminated mare; and intrafollicular oocyte transfer (IFOT), in which immature oocytes are injected into the preovulatory follicle of a mare. ICSI and OT require that the oocytes be mature at the time they are used; both of these techniques have been used successfully for clinical foal production. IFOT can be done with immature oocytes, but it is currently an experimental procedure and should only be considered if there are no laboratory facilities available for oocyte culture to mature the oocytes.

The mare can undergo follicle aspiration for oocyte recovery at a veterinary practice near the mare's location, and then the recovered oocytes can be shipped to an ART laboratory for fertilization. Unlike embryo recovery and transfer, oocyte recovery can be done at any time of the mare's cycle and does not require constant monitoring of the mare's ovarian activity. Oocyte recovery by follicle aspiration is not a benign procedure and has its risks and challenges. For these reasons, mare owners should consider all other options before using oocyte recovery and fertilization methods as a means to produce foals from their mares.

There are two main approaches to recovering oocytes from donor mares: recovery of immature oocytes from all the small follicles on the ovary, and recovery of a maturing oocyte from the one largest follicle produced by the ovary on each cycle, just before it ovulates – the dominant stimulated follicle. Both follicle types can be aspirated using transvaginal ultrasound-guided follicle aspiration (TVA). If your veterinarian does not have access to the equipment needed for TVA, the dominant stimulated follicle can be aspirated by simply placing a long needle through the flank of the mare. These two methods are briefly outlined below.

Transvaginal ultrasound-guided follicle aspiration (TVA)

TVA is performed by veterinarians who are trained in the technique and who have the required equipment. An increasing number of equine reproductive veterinarians are offering this procedure. For TVA, mares are sedated and given pain-relieving drugs during and after the procedure. A long probe with an ultrasound at the tip is inserted into the vagina of the mare. The veterinarian manipulates the mare's ovaries by palpation via the rectum one at a time to bring the ovary near the abdominal side of the vaginal wall. The ovary can be visualized by ultrasound through the vaginal wall. A large needle is then passed through a guide in the probe handle and through the vaginal wall, into each follicle that can be seen on the ovary (or, if only the dominant follicle is being aspirated, into that one follicle). A specialized vacuum pump and supportive media are used to rinse and flush each follicle, while the needle is used to scrape the interior wall of the follicle.

The TVA procedure, especially if only small follicles on the ovaries are being aspirated, involves a long period of manipulation with the veterinarian's hand in the mare's rectum, and passing a needle repeatedly through the wall of the vagina into the abdomen and the ovary. Thus, as with any palpation per rectum, there is a risk for rectal tear. In addition, due to the penetration of the needle into the abdominal cavity, there is a risk for ovarian abscess or hematoma formation, or peritonitis. In addition, adverse drug reactions or other undesired events can occur during the procedure that may cause risk to the health and safety of the mare. These incidents are rare, but can occur, making this procedure one to be considered carefully by mare owners.

Follicle aspiration through the flank

Dominant stimulated follicles are very large (the size of a tangerine), and the oocyte within these follicles is loose from the follicle wall and thus is easily recovered from the follicle. These two attributes make it possible for a veterinarian to recover oocytes from the dominant stimulated follicle by simply placing a needle through the flank of the mare, while the ovary is manipulated via the rectum. The mare is sedated and a small area on the flank is shaved of hair and scrubbed as for surgery. The veterinarian passes a narrow tube (cannula) through the flank muscles, and then passes a long needle through the tube. The ovary with the dominant follicle is manipulated via the rectum and the follicle is pressed against the tube, then the needle is inserted into the follicle and the contents aspirated. The oocyte is recovered from the dominant stimulated follicle about 80% of the time using this method.

Scheduling for oocyte recovery: immature vs. mature follicles

Recovery from immature (small) follicles

Oocytes can be recovered by TVA from immature follicles present on the donor mare's ovaries at any time in the cycle. However, it is recommended to schedule a follicle aspiration at a time when the mare is noted to have a large number of small follicles (between 5 mm and 20 mm in diameter). Unlike the process of checking a mare for traditional breeding or embryo recovery purposes, follicles much larger in size than 25 mm diameter are avoided for immature oocyte aspiration purposes.

The immature oocytes are tolerant of cooling to room temperature, and can be shipped overnight to the ART laboratory at room temperature without any reduction in viability. At the ART laboratory, these oocytes will be placed in culture with hormones to cause them to mature and be ready to be fertilized.

Typically, a total of 10 to 15 follicles may be aspirated from a mare during TVA of immature follicles. More follicles may be available in larger Warmblood-type mares. Oocyte recovery rates can vary based on the skills of the veterinarian performing the procedure and characteristics of the mare. Typically, oocytes are recovered from about 50% of immature follicles aspirated. Then, only about half of the recovered oocytes will "mature" when they are cultured at the ART laboratory and thus be suitable to fertilize. Thus, from a normal Quarter Horse-type mare, this procedure commonly provides three or more mature oocytes per TVA to be used for fertilization attempts.

Oocyte recovery from the stimulated dominant follicle

Because there is only one dominant follicle (or, less commonly, two) per cycle in the mare, recovering oocytes from the dominant stimulated follicle provides many fewer oocytes for fertilization than does aspirating all the immature follicles on the ovaries.

However, certain mares are poor candidates for immature follicle aspiration by TVA. This can be the case of aged mares that develop only a few follicles, mares with uterine infections that make transvaginal manipulation potentially dangerous, maiden mares with extreme sensitivity to the vaginal probe and needle manipulations, or other specialized cases in which either few follicles can be aspirated, or a transvaginal procedure cannot be performed. Recovery of the oocyte from the one large, dominant stimulated follicle allows us the best chance to obtain a mature oocyte in mares that have only one or a few follicles, or to bypass the uterus altogether using the flank aspiration method if no TVA equipment is available, or transvaginal manipulation is not advisable.

To aspirate the dominant preovulatory follicle, the mare's follicle growth is monitored by the veterinarian as if the mare were to be inseminated. When the follicle is large enough, the mare

is given hormones to trigger ovulation, and the oocyte is recovered from the follicle only a few hours before ovulation is scheduled to occur, typically 24 to 35 hours after the ovulatory hormones are administered.

Because this follicle was the one the mare had “chosen” as the best follicle to ovulate, and the oocyte within it underwent maturation within this perfect environment, the oocyte recovered from the dominant stimulated follicle is typically of very high quality. Because the oocyte was near ovulation (was soon to be released from the follicle) it is detached from the follicle wall and is easy to recover – recovery rates on aspiration of stimulated dominant follicles are often around 80%.

Because these oocytes are actively maturing when they are recovered, they are significantly more sensitive to environmental factors than are oocytes from immature (small) follicles. Practitioners will need to keep the aspirated fluid warm after recovery and while searching for the oocyte. When the oocyte is found, a portable incubator is used to keep the oocyte at equine body temperature (~38 °C) during transport.

The oocyte recovered from the dominant stimulated follicle can be fertilized by oocyte transfer to the oviduct of a host mare, or by ICSI. Because this oocyte has already progressed toward a matured state at the time it is removed from the follicle, proper timing of the subsequent fertilization is of utmost importance. Typically, oocyte transfer or ICSI is done 36 to 44 hours after the hormonal stimulus is administered to the host mare. Therefore, the timeframe for shipment and arrival of the oocyte to the designated ART laboratory is much less forgiving than that for immature oocytes. It is also imperative that semen from the desired stallion is shipped to the ART laboratory prior to the shipment of the oocyte.

Oocyte vitrification

While cryopreservation (freezing or vitrification) of equine embryos is successful, and can be used for preservation of genetics and even for reproductive management, cryopreservation of unfertilized equine oocytes is, at the time of this writing, still in its initial stages. Successful embryo and live foal production from vitrified equine oocytes recovered from both live and deceased mares have been reported. However, this technology needs further development before being considered clinically applicable, as the success rates are very low.

Some breed registries have already placed restrictions on the use of vitrified oocytes from donor mares despite the infancy of this practice. Mare owners should be well-informed before committing their mare’s valuable oocytes to this procedure.

E. INTRACYTOPLASMIC SPERM INJECTION (ICSI)

Oocytes harvested from live mares or harvested post mortem can be fertilized in the ART laboratory by the injection of a single sperm into the cytoplasm of the oocyte (Fig. 1). This technique of intracytoplasmic sperm injection (ICSI) is especially useful when numbers of sperm are limited or sperm are of lower quality, since only one sperm is needed per oocyte.

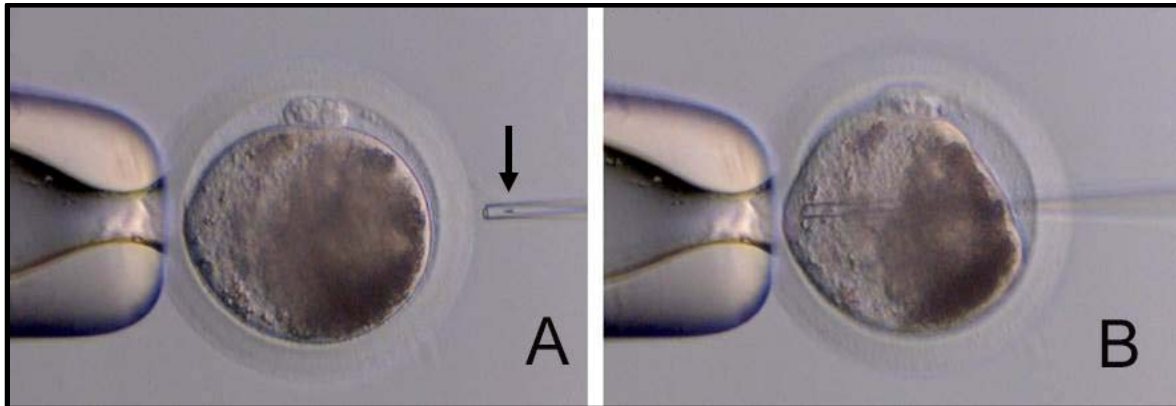


Figure 1. Intracytoplasmic sperm injection (ICSI). A. The sperm (arrow) is picked up in a narrow pipette. B. The sperm is injected into the cytoplasm of the oocyte.

The fertilized oocyte is cultured for 7 to 10 days, to allow embryo development. About 20% of the injected oocytes will develop into embryos (blastocysts) that can be transferred to the uterus of a recipient mare. Typically, about 50% of transferred embryos will result in a successful pregnancy and foaling. The blastocyst can also be vitrified for later use.

The cost of the oocyte maturation and ICSI procedures can add \$1000 - \$2000 or more to the cost of producing a pregnancy – more if no embryos are produced on several cycles. The cost to transfer an embryo to a recipient mare and purchase the pregnant recipient is the same as for standard embryo recovery and transfer, typically ~\$2000-\$5000.

Application of ICSI

ICSI can be used to produce embryos from mares that cannot conceive or carry an embryo long enough for standard embryo recovery to work. Fertile mares may undergo oocyte recovery and ICSI as well, if they are scheduled to be bred to stallions who are offered “by ICSI only” or that have limited breeding contracts. The success rate of equine ICSI continues to increase, and in some areas it is being used instead of embryo recovery and transfer, even with fertile mares and stallions, because of the ease of mare management and an actual increase in efficiency

over that expected from embryo recovery and transfer (more pregnancies achieved per cycle than with embryo recovery and transfer).

The success of embryo production after the oocytes are fertilized by ICSI can be highly variable. We have noticed a reduced rate of embryo production in mares 3 years in age or under and 25 years of age and older. Owners should note that not all oocytes fertilized by ICSI will be viable for long-term embryo development, and multiple attempts may be necessary before a pregnancy is produced from their mare.

Benefits for the mare owner participating in an ICSI program include:

- Mares do not need to be kept under lights prior to the start of breeding season.
- TVA requires significantly fewer palpation checks as mares can simply have the follicles aspirated every two weeks, as long as their follicle numbers are good.
- Multiple oocytes can be recovered at one time, giving the potential for multiple embryos to be produced; whereas, more standard breeding methods typically give a chance at only one embryo per cycle.
- Mares may have follicles aspirated in the non-breeding season or between competitions and training, returning to work more quickly than they would if managed for regular breeding and embryo recovery.
- Follicle aspiration can be performed in between regular embryo recoveries or in pregnant mares up to 150 days of gestation.

While the cost of the technology is decreasing, transvaginal aspiration and ICSI is still an expensive endeavor. Owners should consider the end value of a foal produced by ICSI before utilizing the technology. Typically, the cost for oocyte aspiration is ~\$1000 and may or may not include shipping fees to send the oocytes to an ICSI laboratory for fertilization. Stallion fees and contracts should always be handled ahead of time, and semen should be available at the ICSI laboratory prior to sending the oocytes to the laboratory. Owners should carefully review all contracts from individual ART laboratories before deciding which option is the best fit for their program.

F. OOCYTE TRANSFER TO THE OVIDUCT

Options exist outside of ICSI for production of foals from a mare's recovered unfertilized oocyte. One such procedure is oocyte transfer, whereby oocytes recovered by TVA or flank aspiration from a dominant, stimulated preovulatory follicle (and thus the oocyte is already mature), or recovered by TVA or post-mortem and matured *in vitro*, may be transferred surgically, directly to the oviduct of a recipient mare. The recipient mare is inseminated with semen from the desired stallion. To prevent the recipient mare from conceiving with her own oocyte, the recipient's follicle is aspirated to remove her maturing oocyte. Recovery rate is not always 100%, so availability of multiple synchronized recipients is recommended. Alternatively, a non-ovulating, hormone-treated recipient mare can be used.

Oocyte transfer requires that the recipient mare undergo surgery, in order to place the donor mare's oocyte into the recipient's oviduct (Fallopian tube). This is usually performed as a standing surgery, through an incision in the mare's flank. It is best if only one high-quality oocyte, such as an oocyte recovered from a dominant stimulated follicle, is transferred. In this case, if the recipient mare gets pregnant, she can carry that foal to term. However, in some cases such as recovery of oocytes post-mortem, multiple oocytes must be transferred into the oviduct of one recipient mare. In this case, because a mare cannot carry multiple pregnancies, the uterus of the recipient mare can be flushed for embryo recovery about 8 days after the transfer, and embryos transferred singly into the uteri of secondary recipient mares.

Oocyte transfer avoids the need for ICSI (and thus the need for a sophisticated ART laboratory) and is a viable method of producing foals from isolated oocytes. Oocyte transfer should be considered when ICSI is not available; however, a veterinarian skilled in oocyte and embryo manipulation, as well as in surgery, is necessary. If this procedure is to be used with oocytes recovered from immature follicles, the veterinarian must have access to the proper equipment and supplies to perform oocyte maturation before the oocytes are transferred. Oocyte transfer is a complex process, including donor mare cycle management, recipient mare synchronization, oocyte recovery, oocyte handling and short-term culture, recipient mare insemination, surgery for the oocyte transfer, and post-surgical care of the recipient mare. While each step is not overly challenging, managing the entire process requires planning and skill.

G. INTRAFOLLICULAR OOCYTE TRANSFER

Intrafollicular Oocyte Transfer (IFOT) is the process of injecting immature oocytes into a single dominant, preovulatory follicle of a host (recipient) mare. The oocytes can be injected via a transvaginal ultrasound-guided procedure or through the flank. This technique bypasses the need for ICSI and also for oocyte maturation capabilities. It is still experimental, as the success of the procedure is variable and currently the factors affecting its success are unclear. However, embryos have been obtained from immature oocytes transferred to the follicle, and this method can be considered when the availability of an ART laboratory is limited, or the time frame to transport oocytes to an ART laboratory is such that oocytes would not be viable after transport.

In the technique of IFOT, immature oocytes are recovered by TVA or post-mortem. The oocytes are loaded into a needle, and then transferred into the preovulatory follicle of a recipient mare about 30 hours before the follicle ovulates (the recipient mare is given a hormone injection to induce ovulation before the transfer is performed). The mare is then inseminated normally, and monitored for normal ovulation. Embryos can be recovered by flushing of the uterus non-surgically as for standard embryo recovery, 7 to 9 days post-ovulation. In this case, having several recipient mares synchronized with the donor is advised if multiple oocytes have been transferred. In the best report from this procedure, 7 embryos were recovered on uterine flush after transfer of 15 oocytes to the follicle. Additional embryos may be vitrified for later transfer. However, the majority of IFOT procedures result in no embryos being recovered.

It is important to note that with IFOT, one of the recovered embryos may be from the host mare's own oocyte. Thus, it makes sense to use a host mare from which the foal will be of value. If it is necessary to determine whether an embryo is a result of the donor or recipient oocytes, recovered embryos may be sent for biopsy prior to transfer. Embryos may be sent overnight at room temperature to an ART laboratory for biopsy before being flown counter-to-counter back to the recipient facility for transfer. They can also be vitrified pending genetic testing results. If embryo genotyping is not done, all resulting foals will need to be DNA-typed to determine their parentage.

**II. SUMMARY OF METHODS AND PROTOCOLS FOR
PRESERVATION OF GENETICS FROM ANIMALS
POST-MORTEM**

OUTLINE OF AVAILABLE METHODS FOR GENETIC PRESERVATION POST- MORTEM

If your animal has just died, or must be euthanized, there are many methods that can be used to collect and preserve your animal's genetics. These methods are outlined here, and protocols for performing them are presented in detail in the following section. These protocols for post-mortem genetic preservation are presented because time is of the essence and you may not have the time or means to contact experts to obtain this information if your animal is found dead.

Your horse dies when it loses brain function and the heart stops beating. However, the cells within the horse's tissues can remain viable (capable of functioning) for hours after death. There is no hard and fast rule about how long after death the individual cells can survive, but foals have been produced from ovaries that remained in mares for up to 5 hours after euthanasia, and tissue and sperm appear to have even longer survival. The survival of cells and gametes depends upon the temperature at which the animal's body is held after death; cells will lose viability postmortem much faster at warm temperatures than at cool temperatures. Sperm and tissue samples can be stored in the refrigerator. However, **oocytes (eggs) are damaged if they experience temperatures below about 55 °F (13 °C).**

Methods to consider for preservation of genetics postmortem are:

1. **Harvesting of tissue.** Tissue, such as the connective tissue under the skin or under the gums, or bone marrow from the breastbone, can be taken from your animal, kept cold (not frozen!), and shipped overnight to an Assisted Reproductive Technologies (ART) laboratory. The laboratory will place the tissue in an incubator in a medium that will support growth of the cells that make up the tissue. The cells will divide and eventually new cells will grow out from the tissue into the medium. These cells can then be harvested and frozen in liquid nitrogen and stored indefinitely. The cells can be used in the future to produce a clone of your animal. A summary of equine cloning is presented in **Tissue collection for cloning and the basics of cloning**, page 7.
2. **Harvesting of testes.** If your animal is a stallion, the testicles (testes) and the epididymis (the tissue attached to the testis that contains the tightly coiled, convoluted tube that transports sperm from the testis) can be harvested and sent to an ART or semen laboratory. At the laboratory, sperm will be recovered from the epididymis, and these sperm can be frozen as for sperm collected from a stallion's ejaculate. The epididymis of a normal mature stallion contains billions of sperm, so this can provide frozen sperm for later standard insemination of several mares (standard insemination typically requires hundreds of thousands to millions of sperm). For fertilization within the laboratory (via

ICSI, see **Intracytoplasmic Sperm Injection (ICSI)** page 26), only one live sperm is required to fertilize each oocyte, thus freezing of epididymal sperm in very low doses (“ICSI doses”, see **ICSI doses and cuts, and re-freezing of previously-frozen straws**, page 16) can provide enough sperm post-mortem to use for embryo production via ICSI essentially indefinitely.

3. **Cryopreservation of testicular tissue from the testes** may be recommended if the stallion’s genetics are extremely valuable. Within the testis tissue are the spermatogonial stem cells that generate sperm. It is possible that in the future, methods will be available to use these spermatogonia to generate new sperm from your stallion, or even to implant these spermatogonia in another animal to have that animal make your stallion’s sperm! While these techniques are currently not feasible, this is an active area of research. Testicular tissue may also be used to obtain non-reproductive cells, for cloning purposes, if it is the only tissue that remains after the death of the stallion.
4. **Harvesting of ovaries.** If your animal is a mare, the ovaries can be removed and sent to an ART laboratory for collection of the mare’s oocytes (eggs). As noted above, **oocytes do not withstand cold temperatures** so it is important not to cool the ovaries down to much lower than room temperature (no lower than 55 °F (13 °C)) during holding and shipment (**do not refrigerate**)! However, the oocytes within the ovaries will die more quickly if the ovaries are held at body temperature, so the ovaries should be cooled slowly to room temperature or a little below.

It is best that the ovaries get to the ART laboratory within about 6 hours of the mare’s death. Ovaries received more than 12 to 18 hours after the mare’s death are unlikely to provide viable oocytes. Thus, it is necessary to arrange for counter-to-counter shipment to the ART laboratory if possible. At the laboratory, the oocytes will be recovered from the follicles on the ovaries. Typically, 10 to 15 oocytes can be recovered from one pair of ovaries. The oocytes will be placed in an incubator to mature (become ready for fertilization) and then must be fertilized, so you will have to choose a stallion to which you want to breed your mare, and arrange for the breeding contract, etc., in advance. The reason the oocytes must be fertilized is that currently, cryopreservation (freezing) of unfertilized oocytes is very inefficient. However, embryos resulting from fertilization of oocytes can be cryopreserved effectively.

5. **Cryopreservation of ovarian tissue from the ovaries** may be recommended if the mare’s genetics are extremely valuable. Scattered within the ovarian tissue are thousands of microscopic “primordial” follicles, each containing a juvenile oocyte. It is possible that in the future, methods will be available to stimulate these follicles to grow

within the laboratory and provide mature oocytes. While obtaining usable mature oocytes capable of developing into an embryo from this tissue is not currently feasible, this is an area of active research. Ovarian tissue may also potentially be used to obtain non-oocyte cells, for cloning purposes, if it is the only tissue that remains after the death of the mare.

6. **Recovery of oocytes from isolated ovaries.** After your mare's ovaries have been removed, a skilled person, such as a veterinarian experienced in embryo transfer, with the necessary supplies and equipment, can recover oocytes from your mare's ovaries. The isolated oocytes can then be shipped overnight to an ART laboratory for maturation and fertilization. Shipping the isolated oocytes avoids the 6-12 hour time limit associated with shipment of intact ovaries. This is a great help when a mare is found dead or must be immediately euthanized, and there is no time to arrange rapid shipment of the ovaries to an ART laboratory. While the best results for embryo development after ICSI are found for oocytes received in the ART laboratory by the next day, we have produced embryos from isolated oocytes that were held up in shipment and that did not arrive in the laboratory until 3 days after collection. Again, if oocytes are shipped, they should be shipped at between 55 °F and 72 °F (13 to 24 °C).
7. **Intrafollicular transfer of isolated immature oocytes.** If it is possible to recover oocytes from the ovary, but not possible to send the oocytes to an ART laboratory within the time frame necessary for oocyte viability (within 3 days), then an emergency measure to try to salvage the oocytes' potential for embryo development is intrafollicular oocyte transfer (IFOT). This is transfer of the immature oocytes to the preovulatory follicle of a host mare. The host mare is then inseminated. This is a complex procedure that must be done by your veterinarian, but it can be done with minimal equipment. This procedure has a limited chance of success in inexperienced hands, but it does provide the possibility that an embryo may be produced from your mares' oocytes.
8. **Emergency freezing methods for tissue and sperm.** Cells and sperm are present in very high numbers (millions to billions) in the tissue or epididymis of an animal. If there is no possible way for tissue or testes to be sent to an ART laboratory within 1-3 days, attempting to freeze the tissue or sperm using household materials as freezing media and cryoprotectants (compounds that reduce freezing injury to the cells) may result in at least a few cells surviving. Cloning has been successful in mice using cells from tissue that was frozen without cryoprotection -- these cells would not have grown in culture so they are essentially "dead." However, the cloning was extremely inefficient and required multiple attempts. For sperm, in horses it has been shown that "dead" sperm (sperm that are not moving) and even freeze-dried sperm can be used to successfully

fertilize oocytes by ICSI; however, again, this is inefficient. Thus, if an animal has died and its genetics are irreplaceable, and the above transport methods cannot be performed, these emergency measures are worth attempting and we have provided protocols for performing them.

A. RECOVERY AND HANDLING OF SOMATIC CELL TISSUE FOR FUTURE CLONING

General considerations

1. This protocol is written to explain the procedures for recovery and handling of tissue for the veterinarian that may have limited experience with horses or with reproduction. Because these procedures are applied to animals that might have died unexpectedly, they may need to be conducted without the desired materials and equipment on hand. Thus, we have included “last resort” methods that are not optimal but may be used to attempt to save genetic material when there is no other method available.
2. **Viability.** The somatic (body) tissue should be collected as soon after the animal’s death as possible. The cooler the body is (above freezing), the longer the tissue will survive. However, freezing essentially kills the cells, as ice crystals form within the cells and cause rupture of the cell membranes. When the tissue is collected, it is therefore collected into cold medium (a balanced salt solution that will support the cells) and is kept cold (on ice) during shipment, but is protected from freezing.
3. **Medium for collection.** The medium in which the tissue is placed needs to support the cells. Balanced salt solutions with buffers to maintain the correct pH are typically used. If there is enough time before an animal is euthanized, **cell culture medium** can be shipped from ART laboratories for this use. If not, many veterinarians have access to **embryo holding medium** used for embryo transfer, and this works well. Almost all veterinarians have access to **Lactated Ringer’s solution**, or, less preferred, **normal saline solution**, and this could work if the other media are not available. If either of these last two solutions is used, putting 10% serum or plasma from another horse into the solutions, using sterile technique, will help to keep the cells alive. In emergency situations, it is possible that contact lens solution or rewetting drops (only the type of solutions that can be put into the eye), or as a last resort, milk that has been boiled for 5 minutes then cooled, could be used.
4. **Contamination.** The animal’s skin and mouth are teeming with bacteria and fungi (micro-organisms). However, the tissue beneath the skin or gums is sterile. It is very important that the tissue samples that are collected are not contaminated with micro-organisms from the skin, mouth, handler’s hands, etc. If the micro-organisms are on the tissue, when the tissue is cultured in the laboratory these will grow and produce toxins that will destroy the cells in the tissue. Thus, ideally the skin should be shaved of hair

and washed, and all manipulations done with sterile instruments. Separate sterile instruments that did not touch the skin or mouth should be used to collect the tissue.

Protocol

1. Contact the laboratory to which you are going to send the tissue, so that they can make sure the personnel are available in the laboratory to process the tissue when it arrives.
2. Prepare 3 to 5 small-capacity (5 to 15-mL, i.e. a few teaspoons to tablespoons) sterile tubes or other containers $\frac{3}{4}$ full of the medium to be used. If sterile tubes are not available, plastic bags such as freezer bags can be used; if these have not been previously opened, the inside is essentially sterile. Glass containers can be sterilized by boiling them for 5-10 minutes. If you are boiling, avoid using plastic, as plastic can emit toxins when heated.
3. Refrigerate the containers with medium until use. When ready to use them, place them in an insulated container (e.g., a Styrofoam box) on ice to bring to the location of the horse.
4. Protect the tubes from the light. This is not necessary until the tissue is in the tubes, but a cover will be needed at that point.
5. Sterilize a scalpel (knife), scissors and forceps (tweezers).
6. Before taking the sample, make sure you have something with which to **label the containers**, such as a Sharpie pen or tape and a pen, and also that there is someone ready to open the containers, who has a sterile instrument, such as an injection needle, ready to help get the tissue from the forceps into the tube.
7. We typically take tissue from the gum, or from the under the skin of the neck under the mane, between the front legs or on the abdomen, because this may have had less sun exposure. It is recommended that at least two different areas be sampled, and the tissue grown as two or more separate cell lines. Try to avoid really fatty areas such as the tail head.
8. **Shave** an area approximately 10 x 10 cm (5 x 5 inches) (unless gum), and **scrub and rinse the skin or gum** with gauze pads or paper towels. The best approach is as if for surgery, with a povidone-iodine-based scrub. Scrub the middle of the shaved area, then move circularly out to the “dirtier” areas, and do not take that pad or towel back to the center. Rinse and repeat for a total of three scrubs. Make sure to remove remnants of scrub (soap).
9. If possible, don sterile gloves and maintain sterility; contamination is the most common problem with tissue biopsies.
10. To take the biopsy, make an incision in the skin or gum. Grasp the edges of the incision and evert them so that the tissue underneath is visible. Grasp this tissue with the forceps, and use the scissors to obtain small (size of a large grain of rice) samples of the

tissue, and place each one immediately into the cold cell-culture medium. Large samples are not better, as the cells in the middle of large pieces of tissue die quickly. The assistant, using the needle, should help move the tissue from the forceps into the tube quickly, and should make sure the tissue goes down into the medium. If you think any contamination may have taken place, start with a new tube, and new instruments if necessary.

11. **Bone marrow** samples: It has recently been indicated that cells recovered from bone marrow can make better donor cells than do skin or gum cells. Thus, trying to recover fluid from the breastbone (sternum) of the horse may be worthwhile. To do this:
 - a. Identify the intersternal spaces (soft areas between the individual bones of the breastbone) at about the level of the horse's elbows, by palpating the breastbone with your fingers.
 - b. Clip the hair and clean the skin in this area.
 - c. Attach a long (10-cm, about 4 in) large-gauge (15-G) needle to a 20-to 50-cc syringe – a syringe without the black rubber plunger, such as a syringe used for insemination, is best, as the rubber may be toxic to cells.
 - d. Place the needle through the skin until it makes contact with the surface of the sternebra (the bone of the breastbone). A slow, back-and-forth, rotational movement is used to advance the needle until it is firmly seated in the bone at a depth of 1 to 2 cm (1/2 to 1 inch).
 - e. Aspirate to remove fluid (it will look like blood) from the bone.
 - f. If possible, dilute the fluid 1:1 with a cell culture medium before shipping; if not possible, ship as is (cool to refrigerator temperature and ship on ice, as described below).
12. If taking samples from multiple areas to culture multiple cell lines, put the samples in different tubes and label with the area sampled. Make sure to include **date, horse's name, and owner's name** on the label.
13. Keep the medium and samples **cold (but not frozen)**, and **cover the container** so the tubes are not exposed to sun or fluorescent light. Transport **on ice**, as quickly as possible to the laboratory.

Expected results

1. If the tissue was not contaminated during removal, and the animal died within a few hours of tissue removal, cell culture, harvest and freezing are almost always successful.
2. If the cells are used for cloning, the ability of the cultured cells to support embryo development after the cloning procedure can vary. In our experience, we have performed cloning with 12 different cell lines, and of these, one cell line did not support normal embryo development after cloning.

B. REMOVAL, HANDLING AND SHIPMENT OF TESTES

General considerations

1. This protocol is written to explain the procedures for the veterinarian that may have limited experience with horses or with reproduction. Because these procedures are applied to animals that might have died unexpectedly, they may need to be conducted without the desired materials on hand. Thus, we have included “last resort” methods that are not optimal but may be used to attempt to save genetic material when there is no other method available.
2. Both the testis and **epididymis** (plural, epididymides; the attached sperm-conducting and storage organ; see Fig. 2A, below), can be removed POST-MORTEM OR AT CASTRATION and sent to a semen-freezing laboratory. The epididymal tail is where the more mature sperm, which can be successfully frozen for breeding, are located; therefore, it is critical that this structure is not damaged or lacerated during the removal process. In addition, the ductus deferens, the long tube leading from the epididymal tail, should be ligated (tied off; Fig. 2B) to prevent leakage of sperm during transit.
3. When the horse is dead or under general anesthesia (if being castrated), the skin of the scrotum is cut open, and the testes and epididymides are removed, **taking care to not lacerate the epididymis**, and shipped to the laboratory **at refrigerator temperature**.
4. At the laboratory, sperm are recovered from the epididymis and frozen for future use.
5. For best results, freezing of semen should be completed within 48 hours of tissue harvesting.
6. While sufficient sperm should be available from a mature stallion to allow freezing of standard insemination doses, pregnancy rates when using frozen and thawed epididymal sperm can be low. It is recommended that the owner request that at least part of the sperm that is collected be frozen in straws with a low number of sperm, suitable for use for ICSI (“ICSI doses”; see **ICSI doses and cuts, and re-freezing of previously-frozen straws**, page 16).
7. If a stallion is found dead, but the time of death is unknown, the testes/epididymides should be removed and cooled as presented below. It is unknown how long the sperm can survive at body temperature, but an effort should be made to preserve the sample. The sperm quality will be evaluated prior to freezing and if the sperm are nonviable at that time (that is, the sperms’ outer membranes are no longer intact) it is unlikely they are of any use. However, ICSI doses could still be prepared and frozen in order to evaluate if the sperm are intact enough to fertilize using ICSI.

Protocol

1. Contact the laboratory to which you are going to send the testes/epididymides, so that they can make sure the personnel are available in the laboratory to process the samples and freeze the recovered sperm.
2. If the stallion is to be euthanized, it is probably best to avoid barbiturates prior to removal. Regardless, the comfort of the animal should be a concern at all times.
3. Injection of a local anesthetic into the testes should be avoided since it could accidentally be injected into the epididymis. If a "local" is needed it should be directed high into the spermatic cord area to avoid the epididymis.
4. Open the skin of the scrotum and the external tunic of the testis, as for a castration, and remove the testis and, importantly, the attached **epididymis** and at least 6 inches of the **ductus deferens** (the straight tube extending from the epididymis):

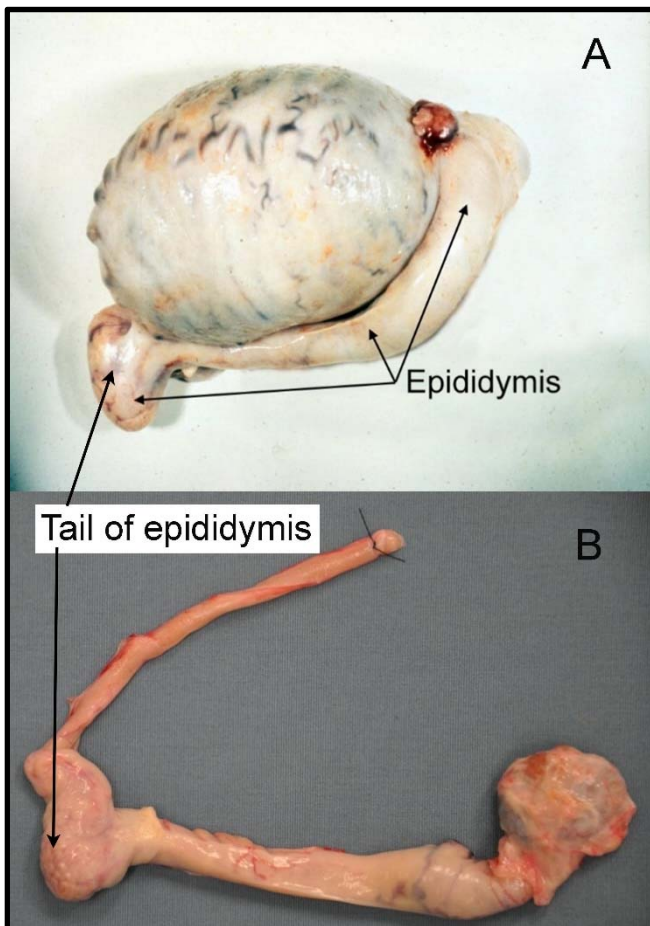


Figure 2. Testis and epididymis. A. Testis with epididymis attached. B. An epididymis dissected from the testis, showing the ductus deferens (arrow) tied with a suture to prevent loss of sperm. In practice, when sending tissue to the ART laboratory for semen freezing, the epididymis should be left on the testis to avoid the chance of lacerating it during dissection. However, the ductus should be ligated as shown.

5. Tie off the end of the ductus deferens with a suture or string, so that sperm cannot leak out. If this is not done, significant sperm loss can occur.
6. Rinse any blood/debris from the testes/epididymides and place into a plastic bag.
7. Holding and shipment should be done at refrigerator temperature, but **DO NOT FREEZE**.
8. Shipment can be done by using an insulated semen shipment container such as an Equitainer, with frozen coolant cans (Fig. 3).



Figure 3. Equitainer with shipping components (www.hamiltonbiovet.com).

9. In some cases the testes/epididymides may be too large to fit into these containers. An alternative is to use a Styrofoam container with an internal volume of at least 12 cubic inches. The bottom can be lined with ice (in plastic bags to prevent leakage) or frozen cold packs that will maintain a cool environment for at least 24 hours. Unlike ovary shipment, sperm can tolerate cool temperatures (i.e. refrigerator, not frozen). It is critical that the container does not reach an elevated temperature prior to processing.
10. Transport as quickly as possible to the laboratory. Sperm may remain viable in cooled epididymides for several days. Unlike ovaries, sperm can be maintained for a longer period of time prior to processing, but every effort should be made to ensure arrival at the processing site within 24 hours.

11. The cost for collection of semen from the epididymis may be around \$500; cryopreservation of the semen can cost about \$500-\$750.

Expected results

1. If the testes/epididymides are received by the laboratory within 24 h, and the appropriate temperature (~4-8 °C, or 40- 46 °F) has been maintained, the epididymal sperm may be as viable as ejaculated sperm. Fertility will depend on the cause of death (acute or chronic) and other factors commonly associated with fertility, such as the age of the stallion, sperm quality and the management of the mares at the time of insemination.
2. The amount of sperm recovered varies considerably, but upwards of 30-40 billion sperm have been recovered from an individual stallion.
3. A “breeding dose” is an approximation based on the sperm quality after thawing (usually based largely on sperm motility). For example, some freezing centers calculate a dose as the amount of sperm needed to provide 250 million progressively motile sperm after thawing.
4. Post-thaw motility is usually effective in identifying very poor quality sperm (they are non-motile), but is less accurate in identifying sperm that may appear good (have good motility) but nevertheless have poor fertility.
5. “ICSI doses” (see **ICSI doses and cuts, and re-freezing of previously-frozen straws**, page 16) may be frozen at 1 million sperm per straw; hundreds to thousands of such doses may be produced.

C. EMERGENCY FREEZING METHODS FOR SOMATIC TISSUE AND SPERM

If it is not possible to send collected tissue or testes/epididymides to an ART laboratory, then emergency measures may be tried in order to freeze somatic tissue or sperm to retain some viability, using household products available in a pharmacy.

General considerations

1. All procedures should be done in as sterile a manner possible, to avoid contamination.
2. Containers and instruments should be boiled for 5-10 minutes, then cooled, before use.
3. The inside of plastic bags is typically sterile; plastic bags should not be boiled as they can release toxins.
4. All medium components should be as free of preservatives and additives as possible.

Freezing medium components

Figure 4. Example of nonfat dry milk powder that can be used for production of a basic emergency freezing extender.



1. Base medium ingredients
 - a. Distilled water (from grocery store).
 - b. Dried skim milk (e.g., Sanalac, Fig. 4) can be purchased at most grocery stores
 - i. If using liquid skim milk, it must be heated to a simmer for 10 min and cooled before use.
 - c. Glucose (dextrose) may be available in grocery stores or pharmacies, otherwise use table sugar (sucrose)
 - d. Fresh egg yolk
 - e. The above ingredients will produce the best “home-made” solution, but if any components are not available, it is possible to use skim milk (simmered gently for 5 min and cooled) as the solution (with cryoprotectant, see below).
2. Cryoprotectant
 - a. Glycerine (glycerol). This is available in most pharmacies
 - b. Automobile antifreeze (propylene glycol) can potentially be used instead of glycerine if no glycerine is available. This must be new (not taken from the car). Use about twice as much propylene glycol as you would glycerine.
3. Conversions for volume 1 teaspoon= 5 mL; 1 tablespoon= 15 mL

4. Formula for freezing medium (recipe can be doubled, tripled, etc., depending on size of container). Combine ingredients and mix gently:
 - 2.4 g Sanalac (**about 2 teaspoons**)
 - 4.9 g glucose or sucrose (**a little more than 1 teaspoon**)
 - 100 mL water (20 teaspoons or 6 tablespoons + 2 teaspoons)
 - 5.0 mL egg yolk (0.8 teaspoon)
 - 3.5 mL glycerine (0.7 teaspoon)

Protocol for somatic tissue

1. Prepare freezing medium using the formula above.
2. Chill the freezing medium to refrigerator temperature.
3. Place the recovered tissue into a flat-bottomed container with enough medium to cover the tissue.
4. Using a new, sterilized razor blade or sharp knife, mince the tissue into the smallest pieces possible.
5. Pour out the medium, and replace it with new chilled freezing medium, or take the pieces and put them into a small container with chilled freezing medium.
6. If possible, divide tissue among multiple small containers, e.g. plastic bags.
7. Place the medium with the tissue pieces into the refrigerator for 20 minutes.
8. Place the tissue into the freezer.

Protocol for sperm

1. Prepare freezing medium.
2. Chill some of the freezing medium in the refrigerator.
3. Cut the epididymis from the testis, and place the epididymis into a flat-bottomed container with enough room-temperature medium to cover the tissue.
4. Using a new sterile razor blade or sharp knife, slice multiple times – 10 to 20 times or more -- across the epididymis, cutting into the underlying tubules to release sperm.
5. Apply pressure to the tissue to encourage sperm to come out from the epididymal tubes.
6. Allow to sit for 5-10 minutes.
7. Remove the tissue.
8. Measure the amount of the sperm-containing medium.
9. Slowly add an equal volume of the chilled freezing medium to the container with the sperm to dilute the sperm further.
10. Divide sperm solution into multiple small containers, e.g. plastic bags.
11. Place the containers of sperm solution into the refrigerator for 20 minutes.
12. Place sperm solution into the freezer.

D. CRYOPRESERVATION OF TESTICULAR TISSUE FROM THE TESTES

If the stallion's genetics are extremely valuable, you can request the ART laboratory to cryopreserve tissue sections from the testis. Within the testis tissue are the spermatogonial stem cells that generate sperm. It is possible that in the future, methods will be available to use these spermatogonial stem cells to generate new sperm from your stallion, or even to implant these cells in another animal to have that animal make your stallion's sperm.

While these techniques are currently not clinically feasible, studies have shown that they are possible, and this is an active area of research. Testicular tissue may also potentially be used to obtain non-reproductive cells, for cloning purposes, if it is the only tissue that remains after the death of the stallion.

E. REMOVAL, HANDLING AND SHIPMENT OF OVARIES

General considerations

1. This protocol is written to explain procedures for the veterinarian that may have limited experience with horses or with reproduction. Because these procedures are applied to animals that might have died unexpectedly, they may need to be conducted without the desired materials on hand. Thus, we have included “last resort” methods that are not optimal but may be used to attempt to save genetic material when there is no other method available.
2. Contact the laboratory to which you are going to send the ovaries, so that they can make sure the personnel are available in the laboratory to process the ovaries when they arrive.
3. Equine oocytes do not tolerate temperatures less than about 13 °C (55 °F), therefore **DO NOT PLACE THE OVARIES ON ICE OR IN REFRIGERATION.**
4. Time is of the essence; if possible, try to arrange a euthanasia and transport schedule so that the ovaries arrive at the ART laboratory preferably within 6 hours of the mare’s death.
5. If the ovaries cannot be shipped to the ART laboratory within 12 hours, consider having a local veterinarian recover the oocytes from the ovaries, and ship the isolated oocytes (see **Recovery of oocytes from harvested ovaries**, page 53). Isolated oocytes can be shipped overnight with no detrimental effects on oocyte quality.
6. Removal of the ovaries will require a large incision into the abdomen of the mare after death. This will make it difficult to move the mare’s body afterward, as abdominal organs may exit through the incision. This can be addressed by taking the body to the site of disposal before harvesting the ovaries, if such transport can be done quickly, or by sewing (suturing) the incision after the ovaries are removed.
7. The oocytes recovered from ovaries removed from mares post-mortem can be used to produce embryos and foals via:
 - a. Fertilization *in vitro* (in the laboratory), typically by ICSI (intracytoplasmic sperm injection) in selected equine ART laboratories.
 - b. Oocyte transfer: The oocytes are matured *in vitro* and then transferred to the oviduct (Fallopian tube) of a live mare (see **Oocyte transfer to the oviduct**, page 28).
 - c. Fertilization *in vivo* (in a live mare), by transferring the immature oocytes into the preovulatory follicle of a host mare (IFOT), and then inseminating the mare by standard artificial insemination. This is inefficient but has worked sporadically, and can be used as a last resort if no laboratory facilities or only limited

laboratory facilities are available (see **Intrafollicular transfer of isolated oocytes**, page 60).

- d. In all cases, **semen from the stallion of your choice must be available** to the laboratory or veterinary practice the day after the oocytes are received. For oocyte transfer to a live mare, a standard insemination dose of fertile semen is required. Start arranging this as soon as the ovaries are packaged.

Protocol for ovary removal

1. If the mare is alive but will be scheduled for euthanasia in the future, have your veterinarian contact an ART laboratory regarding the best methods for euthanasia, ovary shipment scheduling, etc. Necessary flight arrangements for ovary shipment should be made ahead of time, and the time of euthanasia coordinated to provide the shortest shipment time possible.
2. If the mare is alive but must be *immediately* euthanized by the veterinarian, contact the ART laboratory to which the ovaries will be transported, to ensure that the laboratory has the personnel available to process the ovaries, and to try to arrange the best schedule for transportation of the ovaries.
3. Our recommended method for recovering the ovaries from mares to be euthanized by the veterinarian is to anesthetize the mare with ketamine and xylazine, remove the ovaries, then euthanize the mare.
 - a. Typical doses used are xylazine, 1-2 mg/kg i.v. (500 to 1000 mg per 500-kg horse), followed 5 minutes later by ketamine HCl, 2-3 mg/kg i.v. (1000 to 1500 mg per 500-kg horse).
 - b. Following ovary removal the mare is euthanized with KCl or commercial euthanasia solution. Euthanasia solution should be drawn up in the appropriate amount before the mare is anesthetized. If the mare becomes in any way responsive during ovary removal, the euthanasia solution should be immediately administered, then the ovary removal completed.
4. If the mare is alive but needs to be immediately euthanized by the owner, gunshot is compatible with preserving the ovaries. Non-veterinarians should not attempt to work with the mare until it is clear that the mare is dead. This includes the mare having no heartbeat, pulse or breathing, and not reacting to a finger placed on the eyeball.
5. Materials needed are
 - a. Palpation sleeves and surgical gloves, if available.
 - b. A clean scalpel or knife, to make the incision.
 - c. A sterile container (plastic bag is fine if not previously opened) to hold the ovaries. No medium (fluid) is necessary in the bag.

- d. An insulated, e.g. Styrofoam container that already contains a bag of water (double-bagged to prevent leaking) at body temperature (37 °C or 98 °F). This will initially keep the ovaries warm so that they cool slowly to room temperature.
6. Once the mare is completely anesthetized or is dead, approach the mare – she can be lying on either side. Both ovaries can be removed from one side, but this is difficult for the inexperienced operator. Therefore it is good to be prepared to roll the mare over to the other side.
7. If possible, shave the hair from the area of the flank, and/or clean this area (white area, Fig. 5):

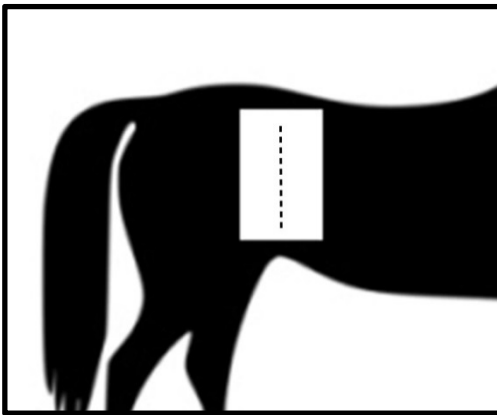


Figure 5. Location of incision to remove ovaries from a mare after death.

8. If possible, put on palpation sleeves on both arms, and don surgical or exam gloves over the sleeves.
9. Using a scalpel or sharp knife, make an incision completely through the abdominal muscles (see dotted line in above drawing).
10. Continue deepening the incision until the abdominal cavity has been entered.
11. To locate the ovary closest to the incision, identify the uterine horn and follow the horn to the ovary. Alternatively, sweep the hand along the muscles on the roof of the abdomen, backward into the pelvic cavity. The ovaries are suspended by a short pedicle (stem) from the body wall (Fig.6).

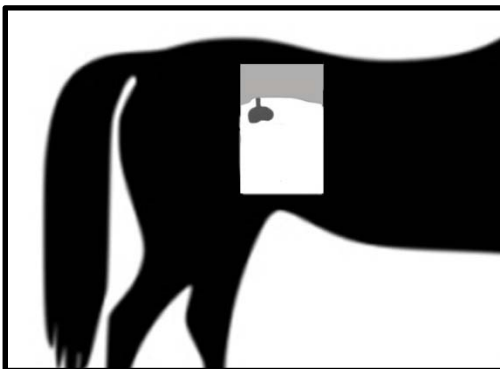


Figure 6. Location of the ovaries within the abdominal cavity. They are suspended from the dorsal body wall (roof of the abdominal cavity) by a short pedicle.

12. The ovary is a solid, very firm structure typically about the size of a golf ball to a small orange. Horse ovaries have a distinctive “kidney shape.” The indentation (ovulation fossa) can be felt with the fingers to confirm that the structure is an ovary.
13. Bring the ovary to the incision and pull very hard on the pedicle to tighten it. The ovary may not come completely out of the incision. Locate the uterine horn attached to the ovary and try to secure it or have someone else hold it; this can be used to help you find the other ovary.
14. Cut through the taut ovarian pedicle. **TAKE CAREFUL PRECAUTIONS NOT TO CUT YOURSELF** while cutting through the tightened pedicle. Cut farther away from your hand than you think is necessary.
15. The removed ovary should be placed into a palpation sleeve or plastic bag (no fluid is needed in the bag) and placed into the Styrofoam container that already contains the 37 °C bag of water (ballast), and the container covered.
16. If you are removing both ovaries through the one incision, and you have kept hold of the uterine horn, the opposite ovary can be located by tracing your hand along the uterine horn from the first ovary to the second horn and ovary. If you do this, you can pull the second ovary cleanly to near the original incision.
17. Pull extensively on the second ovary to get it as close to the incision site as possible; however, this ovary typically must be removed blindly, with the hands in the abdominal cavity. **TAKE EXTREME PRECAUTIONS NOT TO CUT YOURSELF** while removing this ovary.
18. If the uterine horn was not secured, or if the second ovary is difficult to locate, the second ovary may only be able to be grasped through the mesentery (the thin tissue suspending the intestines). Pull the ovarian pedicle taut and cut through it; the ovary can be removed even though it seems that the mesentery is not being cut.
19. Alternatively, roll the mare over and remove the opposite ovary in a similar manner to the first one.
20. Place the second ovary in the same bag as the first. Seal the bag with a minimum of air in it, and place the bag in the insulated container.
21. DO NOT PUT OVARIES ON ICE OR IN REFRIGERATOR.

Protocol for ovary handling and shipment

1. The purpose of the body-temperature ballast (bags of water) in the Styrofoam container is to prevent the ovaries from cooling too rapidly. The ballast, as well as the ovaries, should cool to room temperature slowly.
2. To ship the ovaries, an Equitainer, made for shipping equine semen, or an EquOocyte, made for shipping oocytes, works well. If such containers are available:

- a. Place coolant cans at the temperatures outlined below (dependent on the duration of shipment) into the central cavity of the container.
 - b. Remove the bag containing the ovaries from the Styrofoam container and place it in the holder (isothermalizer) of the container.
 - c. If ovaries do not fit inside the isothermalizer, the bag containing the ovaries can be wrapped in additional padding, such as bubble wrap, paper towels or foam padding, and placed directly on top of the cans in the container.
 - d. Close and latch the container.
3. If an Equitainer or EquiOocyte is not available, additional packaging can be placed in the Styrofoam container and the ovaries shipped in this container. Add more bags filled with water (bags of saline work well for this) to the container, at the temperatures outlined below (depending on duration of shipment). Enclose the Styrofoam container in a cardboard box (necessary for counter-to-counter shipments).
4. **The temperature of shipment** is dependent upon the expected duration of shipment. All efforts should be made to get the ovaries to the ICSI laboratory within 12 h of the mare's death, and preferably within 6 h:
 - a. **If shipment time is less than 6 hours**, ship with all materials starting at room temperature, except some ballast immediately around the ovaries that has been cooling with the ovaries since they were collected. This will allow the ovaries to continue to cool slowly to room temperature.
 - b. **If shipment time is expected to be more than 6 hours**, the goal is to slowly cool the ovaries to 55-65 °F (13-18 °C) during shipment. This can be done in an Equitainer by using one frozen can on the BOTTOM and one room temperature can on top of it, closest to the isothermalizer. If using a Styrofoam container, ballast cooled to ~13 °C (55 °F) can be used. The ovaries are placed in the container with a small amount of room temperature ballast around them.
5. Include in the shipping container all the important information about the mare, including the reason for euthanasia, how long the mare was sick before euthanasia, what treatments she received, the method of euthanasia, the time the ovaries were removed, as well as the mare's name, owner's address and contact information.

Expected results

1. The number of viable oocytes recovered from a mare's ovaries is variable and may depend on factors including the age of the mare or the stage of her cycle at the time of her death, the length of time she may have undergone treatments for illnesses, lameness, or other debilitating issues, and the drugs given at or just before her death.
2. Typically, 10 to 15 oocytes are recovered from a pair of ovaries post-mortem.
3. About 50% of the oocytes should mature in culture to be fertilized by ICSI.

4. Overall, this means that if ICSI is conducted with the oocytes, based on results for post-mortem ICSI at the Equine Embryo Laboratory at Texas A&M University (2010-2015), an average of 1.3 blastocysts is expected per mare, with an average foal production of 0.54 foals per mare. Essentially, this means that ON AVERAGE the owner has a 50:50 chance of getting a foal from the procedure.
5. The likelihood of embryo and foal production is dependent on many factors including the time of ovary removal (before or after death), the age of the mare, the mare's illness, medications received, and the time and conditions of ovary transport.

F. CRYOPRESERVATION OF OVARIAN TISSUE FROM THE OVARIES

If your mare's genetics are extremely valuable, you may request that the ART laboratory cryopreserve sections of the mare's ovaries. Scattered within the ovarian tissue are thousands of microscopic "primordial" follicles, each containing a juvenile oocyte. It is possible that in the future, methods will be available to stimulate these follicles to grow within the laboratory, and to provide mature oocytes.

While obtaining usable mature oocytes capable of developing into an embryo from this tissue is not currently feasible, this is an active area of research. Ovarian tissue may also be used to obtain non-oocyte cells, for cloning purposes, if it is the only tissue that remains after the death of the mare.

G. RECOVERY OF OOCYTES FROM HARVESTED OVARIES

General considerations

1. If the ovaries cannot be transported to an ART lab for oocyte recovery within about 12 hours of the death of the mare, veterinarians with some expertise in embryo recovery and transfer may attempt to recover the oocytes so that they may be shipped to the laboratory by overnight courier.
2. Contact the laboratory to which you are going to send the oocytes, so that they can make sure personnel are available in the laboratory to handle the oocytes when they arrive.
3. Ovary dissection and oocyte recovery are done at ROOM TEMPERATURE.
4. The oocytes should be shipped at room temperature or down to 13 °C (55 °F); DO NOT PLACE THE OVARIES OR OOCYTES ON ICE OR IN REFRIGERATION.
5. Isolated oocytes may be shipped overnight with no detrimental effects on viability, and embryos have been produced from oocytes held for up to three nights before being received.
6. The oocytes will be matured by the ART laboratory and then must undergo fertilization (typically by ICSI). This means that semen from the stallion of your choice must arrive at the laboratory the day after the oocytes arrive. Start arranging this as soon as the oocytes are packaged.

Protocol for oocyte recovery

1. The set-up of the ovary processing station should be done with caution toward cleanliness. If possible, a sterile drape should be laid down and all materials gathered on the drape.
2. Recommended materials include: sterile gauze, small collection dishes such as 35-mm petri dishes, scalpel and handle, bone curettes size 0 and 2, surgical scissors, hemostats, 20-mL all-plastic syringe (avoid using syringes with black rubber as they may be toxic to oocytes), 20-gauge needle, and embryo holding medium or (less desirable) embryo flush medium (Fig. 7).



Figure 7. Supplies needed for post-mortem ovary processing.

3. Place an ovary on a pad of sterile gauze, and remove the excess tissue with scissors or scalpel (Fig. 8).

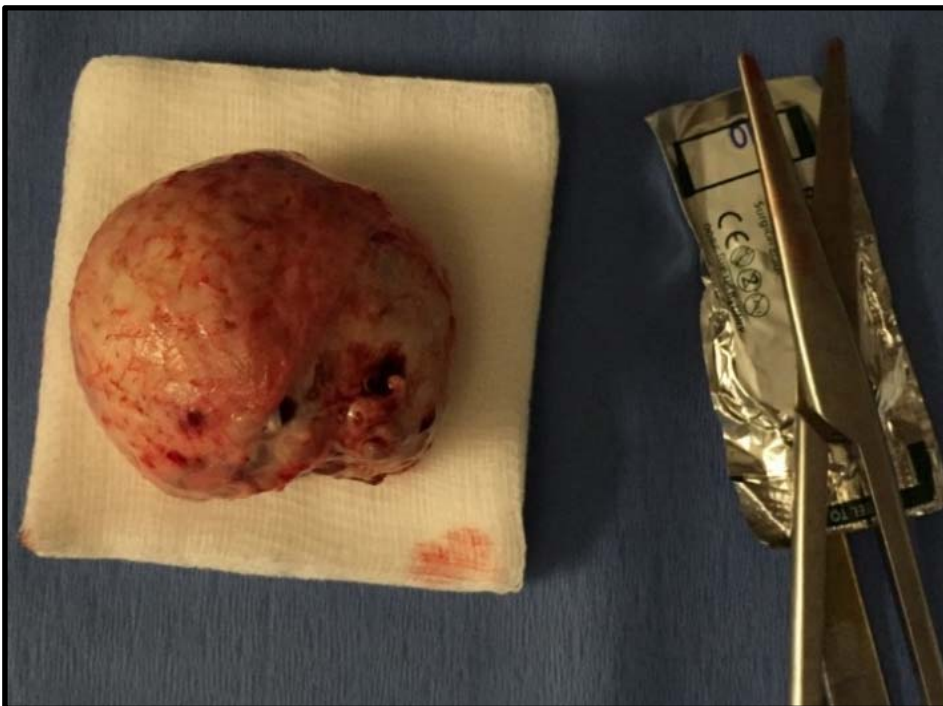


Figure 8. Ovary after excess tissue has been removed.

4. Locate any follicles close to the surface of the ovary, and open them completely (until the inside of both complete halves of the follicle can be seen) with the scalpel (Fig. 9). Follicular fluid will drain from the follicle, but the normal oocyte is attached to the inner walls of the follicle and so will not be lost with this fluid.
5. Using the bone curette or other scraping device, scrape one swath along the inside wall of the follicle (Fig. 9).

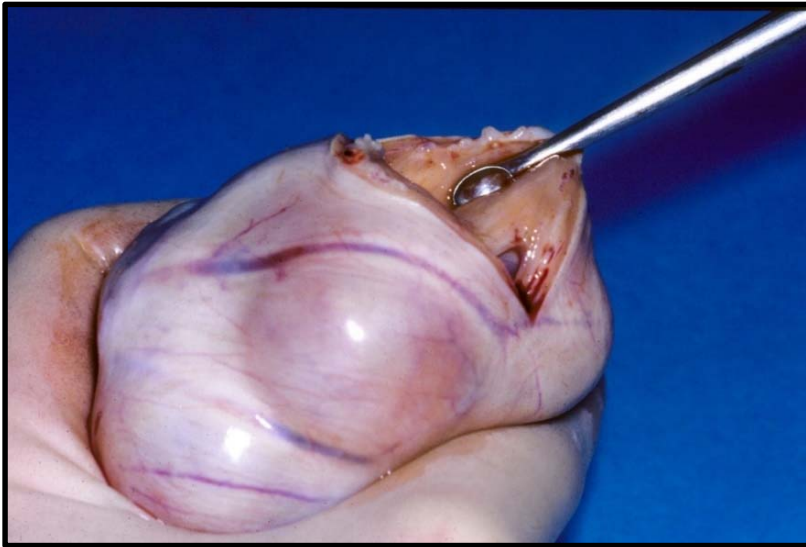


Figure 9. Scraping the inside of the follicle wall with a bone curette.

6. Wash the accumulated tissue into a Petri dish using medium in the syringe and needle (Fig.10).

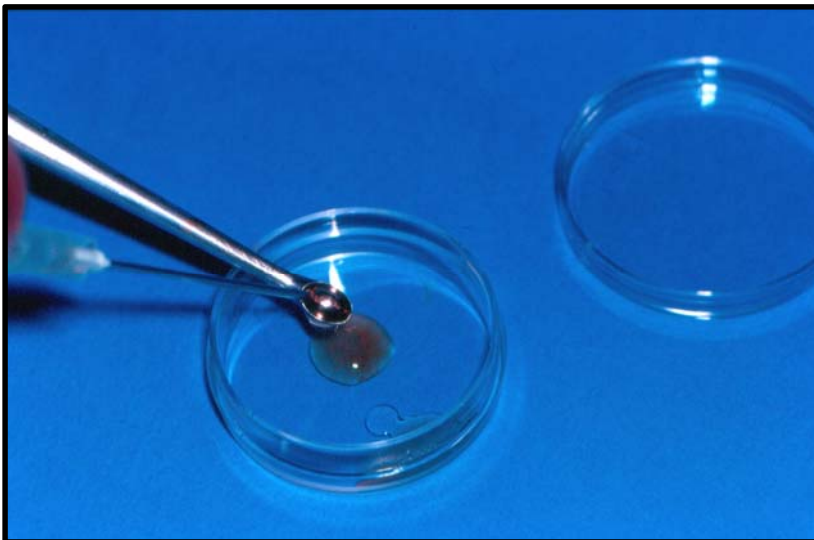


Figure 10. Rinsing the curette into a small petri dish.

7. Continue scraping one swath at a time and rinsing the accumulated tissue into a Petri dish until the entire inner surface of the follicle has been scraped and no more tissue is recovered. For post-mortem ovaries, we scrape and examine one follicle at a time to maximize the number of oocytes recovered.
8. Examine the Petri dish with the accumulated tissue under the stereomicroscope at 40X (or more) to locate the oocytes. Oocytes are typically enclosed in cumulus cells, so they do not look spherical as do equine embryos recovered from the uterus. They may be broken free of the granulosa, or may be in a sheet of granulosa cells (Fig. 11).

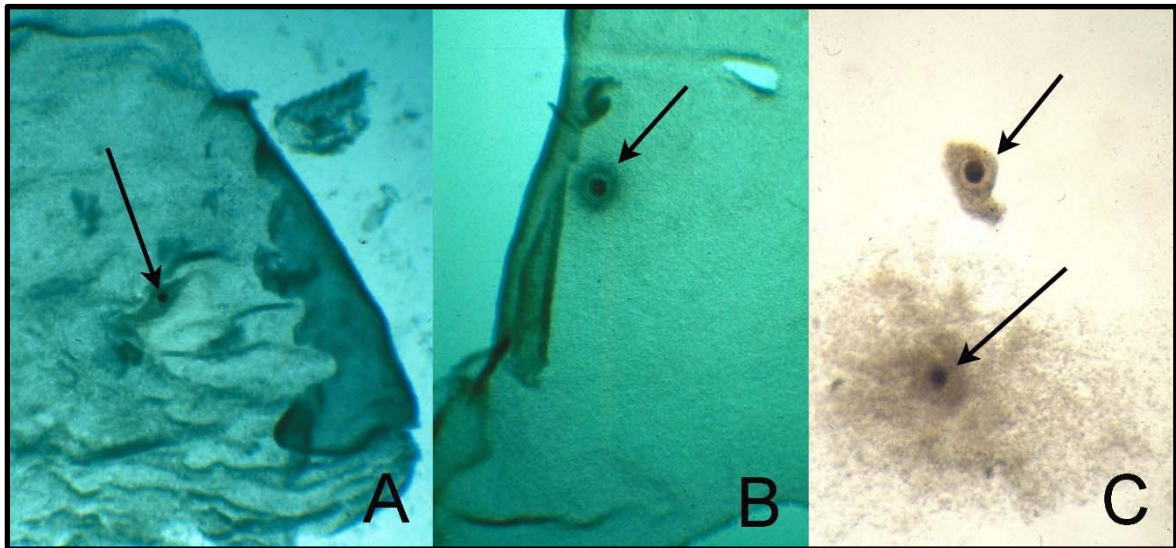


Figure 11. Cumulus-oocyte complexes (arrows) at lower (A) and higher (B, C) magnification. The oocyte-cumulus complex may be found in sheets of granulosa cells (A, B) or may be separated from the granulosa (C) with either compact cumulus (upper complex) or expanded cumulus (lower complex).

9. Some oocytes may have to be carefully separated from large sheets of cumulus cells using 20-gauge needles (Fig. 12).
10. All located oocytes should be moved as they are found into a holding dish containing embryo holding media.

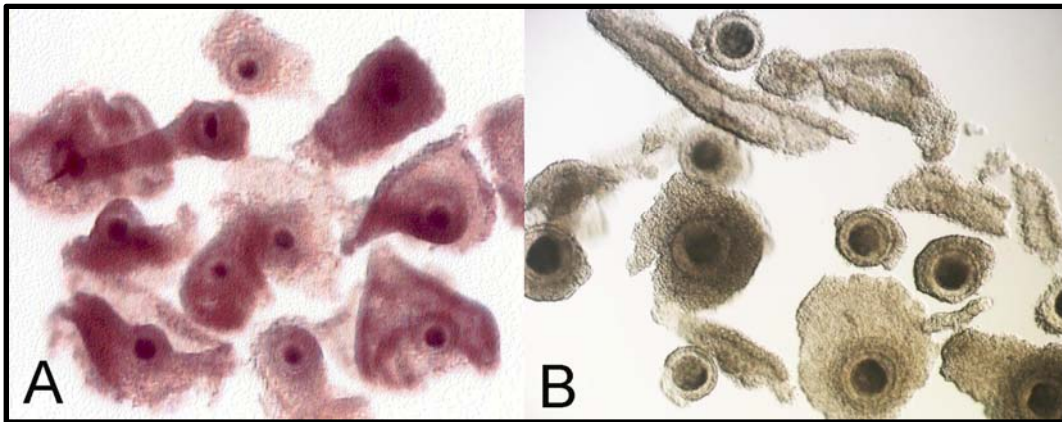


Figure 12. Isolated cumulus-oocyte complexes, and some granulosa tissue (linear pieces of tissue in B) at lower (A) and higher (B) magnification. The cumulus has been cut with needles to a fairly standard size.

11. If equipment to scrape the follicle walls is not available, an 18-gauge needle attached to a syringe or vacuum pump can be used to similarly scrape the tissue from the follicle wall (Fig. 13).
 - a. In this case, the needle is inserted into a container of holding medium between scraping motions to rinse tissue up into the syringe or holding vessel.
 - b. The recovered fluid and tissue is expressed into a Petri dish to search for the oocyte (if syringe is used), or is filtered through an embryo filter to collect oocytes (if vacuum and holding vessel are used).

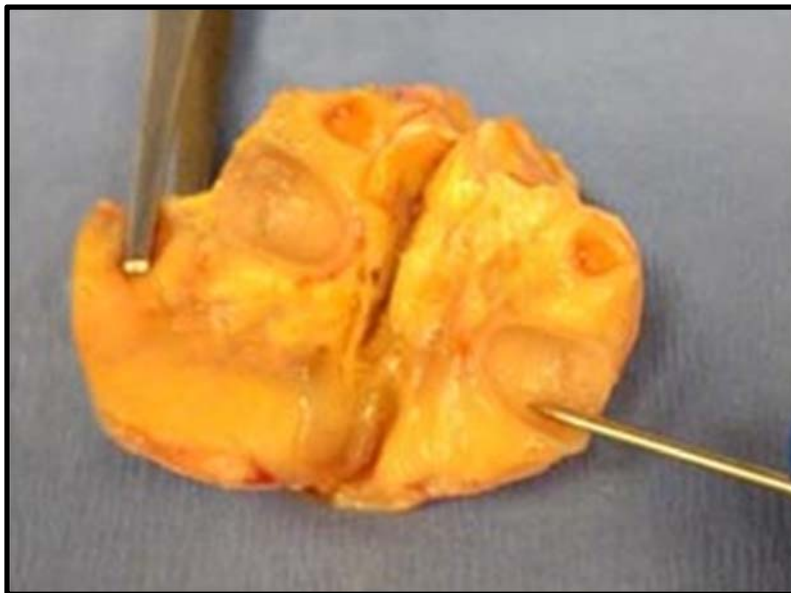


Figure 13. Scraping follicular wall using an 18-gauge needle.

Protocol for shipment of isolated oocytes

1. All procedures should be performed as cleanly as possible to prevent contamination of the oocytes or tube/vial (including the top of the tube or vial).
2. Rinse all collected oocytes through three clean dishes of holding medium, if possible.
3. Fill a sealable tube or vial (1 to 5 mL) about $\frac{3}{4}$ full with holding medium.
4. Label the vial with the date, mare's name, and number of oocytes.
5. Transfer the oocytes into the vial using a micropipette set to 15 μ l, a sterile Pasteur pipette, or other embryo pipette.
6. Add medium until vial is almost full (leave room for cap).
7. Carefully cap the vial using hemostats to prevent your finger from contacting the part of the lid that seats inside the vial.
8. Check under the microscope that all oocytes are in the vial.
9. Wrap cap and top of vial with parafilm, if possible.
10. A semen shipment container can be used but cans must be at ROOM TEMPERATURE or a bit cooler (55-72 °F, or 13-24 °C), not cold.
11. An insulated container such as a Styrofoam box can be used if necessary; use ballast (e.g. bags filled with water and double-bagged) at room temperature or cooler (55-72 °F, or 13-24 °C).
12. The vial should be wrapped in tissue to avoid light exposure, and placed in the shipping container. The vial should be laid on its side to make sure that all the oocytes do not fall into the same area of the tube. Larger tubes (e.g. 5-cc round-bottom tubes) can be shipped upright. There should be lots of padding (tissues or paper towels) to avoid the tube being jostled too much during shipment.
13. A paper with all the mare's information, number of oocytes, and owner contact information should be included in the shipping container.
14. The container should be shipped at priority overnight or counter to counter to the desired ART lab.

Expected results

1. The number of viable oocytes recovered from a mare's ovaries is variable and may depend on a number of factors, including the age of the mare or the stage of her cycle at the time of her death, the length of time she may have undergone treatments for illnesses, lameness, or other debilitating issues, and the drugs given at or just before her death.
2. Typically, 10 to 15 oocytes are recovered from a pair of ovaries post-mortem.
3. After being received by the ART laboratory, about 50% of the oocytes should mature in culture to be fertilized by ICSI.

4. Overall, this means that if ICSI is conducted with the oocytes, based on results for post-mortem ICSI at the Equine Embryo Laboratory at Texas A&M University (2010-2015), an average of 1.3 blastocysts is expected per mare, with an average foal production of 0.54 foals per mare. Essentially, this means that ON AVERAGE the owner has a 50:50 chance of getting a foal from the procedure.
5. The likelihood of embryo and foal production is dependent on many factors including the age of the mare, the mare's illness, medications received, time of ovary removal (before or after death), the time and conditions of oocyte recovery, the media used and method of packaging, and the time and conditions of oocyte transport.

H. INTRAFOLLICULAR TRANSFER OF ISOLATED OOCYTES

General considerations:

1. If oocytes have been recovered from a mare post-mortem and it is not possible to ship the oocytes to an ART laboratory, it may be possible for a veterinarian experienced in reproduction to attempt intrafollicular oocyte transfer (IFOT) as a measure to try to obtain foals from the recovered oocytes. The likelihood of success is not high, and factors influencing the outcome are not clear, but donor mare embryos have been harvested from the host mare's uterus after transfer of immature oocytes to the preovulatory follicle. The following is a method based on several reports of success using this procedure.
2. An apparatus for delivering the oocytes into the follicle is needed. Delivery can be performed by three methods:
 - a. Flank: Through the abdominal wall (see Fig. 14, below); the ovary containing the preovulatory follicle is held against the internal aspect of the wall with the hand via the rectum. For this, a long needle, about 8 in (20 cm) is required. If available, a trochar-cannula, such as an equine cecal cannula, can be placed through the muscles first; this helps to prevent "coring" of the musculature by the needle when it is passed through the wall.

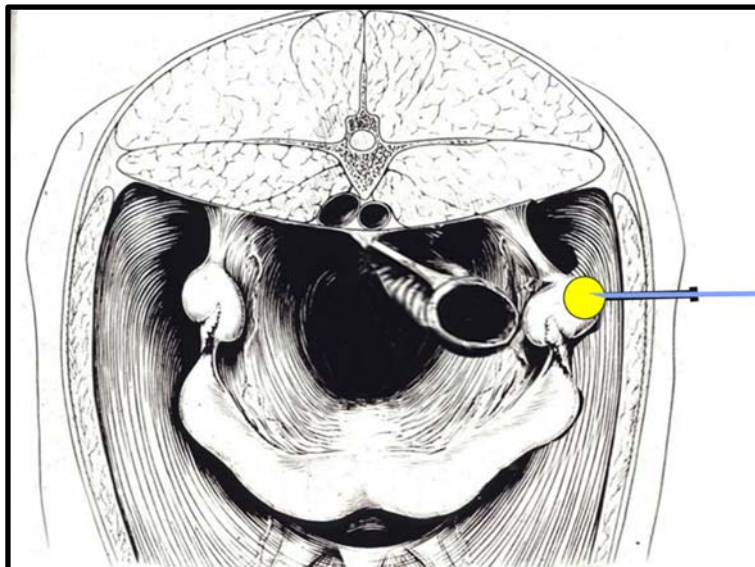


Figure 14. Cross-section through the mare's body showing the position of the ovary and preovulatory follicle during puncture through the flank for intrafollicular oocyte transfer. The ovary is held against the cannula by palpation per rectum. (adapted from Roberts, 1986).

- b. Veterinarians interested in offering or utilizing this technique through the flank may wish to invest in the following tools:
 - i. Disposable Two-Part Trocar Needle set (Cook Medical, Bloomington, Indiana)
 - ii. 18 to 20-gauge, 8-inch needle (Cook Medical, Bloomington, Indiana)
 - iii. Extension set, 38-50 cm (Baxter Healthcare Corporation, Deerfield, Illinois)
 - c. Via transvaginal ultrasound-guided follicle puncture. For veterinarians who already have an equine transvaginal oocyte aspiration set-up, an 18 to 20-gauge ovum aspiration needle (Cook Medical) is used instead of the standard 12-gauge double-lumen needle. Some of these items may be borrowed on an emergency basis from nearby bovine IVF practitioners.
 - d. Via standing flank laparotomy, or if necessary, flank laparotomy under general anesthesia.
3. A suitable host mare will need to be located at the time of the donor mare's death or shortly after determining that immature oocytes recovered from the ovaries will not be able to be shipped to an ICSI laboratory. Desirable recipient mares should be in estrus with a dominant follicle between 33 to 40 mm in diameter.
 4. Semen from the desired stallion will need to be of good quality and a standard insemination dose should be ordered as soon as possible, with preference to cooled-shipped or fresh semen.
 5. Depending on the size of the follicle of the recipient mare, the immature oocytes may be transferred immediately to the follicle after they have been recovered from the deceased mare's ovaries, or the oocytes may be held overnight at room temperature for transfer the following day. Recipient mares are expected to ovulate between 36-40 hours after administration of ovulatory hormones.

Procedures:

Laboratory preparation

1. NOTE: It appears that the equine follicle reacts negatively to bovine albumin. If at all possible, use medium with another source of macromolecule, e.g. PVA, hyaluronan, equine serum, equine follicular fluid, etc. The medium can be tissue culture medium, if this is available, such as TCM199 with Hanks' salts, or it can be embryo holding medium

or embryo flush medium, or, if no other media are available, Lactated Ringer's or normal saline.

2. Make sure that all media used are sterile. Filter through a 0.2-micron filter if possible.
3. After recovering the oocytes from the excised ovaries of the deceased mare, (see **Recovery of oocytes from harvested ovaries**, page 53), place the oocytes into a holding dish in clean medium.
4. Alternatively, hold oocytes overnight at room temperature and then place them into a holding dish in clean medium.
5. Rinse the oocytes through an additional 3 dishes of clean medium and leave them at room temperature.
6. Have 10 mL of media set aside in a sterile tube at room temperature.
7. Using sterile technique, prepare the oocyte delivery apparatus. Below are recommended sizes of needles, etc., but use the closest material you have. The smaller the gauge needle, the smaller the hole in the follicle (so the follicle has less insult, and the oocytes are less likely to leak out of it) and the less medium is needed, so the follicle is not over-inflated. Larger-gauge needles will have less capillary action and will be more likely to allow the oocytes to fall out of the needle after loading. Holding the needle with the tip up will help avoid having the oocytes leaking out.
 - a. Flank: Connect an 18-ga 8-in needle to short (6-20 inches) extension tubing. Place a 5-mL all-plastic (no rubber plunger) syringe on the tubing.
 - b. TVA: Connect syringe and tubing to the 20-ga transvaginal needle.
 - c. Laparotomy: Connect syringe and tubing to a 21-ga 1.5-in needle.
8. Carefully insert the needle into a dish/tube of holding media (without oocytes) and draw back to fill the entire line and 3 mL in the syringe with fluid.
9. Push fluid out of the syringe through entire system - leave plunger at the 1-mL mark.
10. *Loading Oocytes (to be performed when host mare is prepared for the transfer):*
 - a. Have a second operator handling the syringe. The first operator views the dish with the oocytes in it under the stereomicroscope.
 - b. Depending upon the size of the needle (larger gauge needles require more volume), pull 50 to 500 μ l air while the needle is held in the air.
 - c. Pull 100 to 500 μ L of medium by holding needle in the medium away from the oocytes.
 - d. Load oocytes (usually takes about 100 μ l) by applying constant slow suction with syringe while vacuuming up oocytes.
 - e. Pull 50 to 500 μ l of medium following oocytes.
 - f. Pull a small amount of air into the needle.
 - g. Cap needle.
 - h. Carefully replace needle into sterile packaging to be taken to mare-side for transfer.

Mare-side preparation

1. The host mare should receive the ovulatory agent (e.g. deslorelin or hCG) 6 to 12 hours before the transfer.
2. Restrain host mare in stocks and tranquilize the mare (if procedure is to be performed standing: Flank, TVA, standing flank laparotomy). Alternatively, anesthetize mare (laparotomy).
3. For standing follicle puncture, we utilize:
 - a. 2-5 mg detomidine i.v.
 - b. Then immediately before the follicle is to be punctured:
 - A. 5 mg butorphanol iv.
 - c. 120 mg scopolamine butylbromide (Buscopan) i.v.
4. Perform ultrasonography of the ovaries per rectum to be sure that the follicle is still present. Estimate the location of the dominant follicle on the ovary and identify any other larger follicles nearby. Clean rectum of all feces possible.

Flank approach:

1. The operator palpates the mare via the rectum using the hand OPPOSITE to the side of the preovulatory follicle, e.g. right ovary -- left hand.
2. Move the ovary containing the preovulatory follicle to the inner surface of the lateral abdominal wall.
3. With the non-palpating hand, identify the best spot on the external flank to place the needle so that the needle will go directly horizontally into the dominant follicle.
 - a. Clip a small area of hair around the identified spot, and scrub as for surgery.
4. The operator again puts on a rectal sleeve on the palpating hand. Cut the fingers off the sleeve before donning, and place a surgical glove over the hand; this will give better feel on palpation
5. The operator should don a sterile glove on the other hand.
6. Administer the butorphanol and buscopan to the mare.
7. The operator again palpates the mare per rectum and moves the ovary to indicate to the helper where on the flank to place a lidocaine bleb (e.g. 1-2 mL of 2% lidocaine).
8. After the bleb is made, a stab incision is made with a scalpel into the bleb. Typically this is done by the operator palpating the mare – leaning around to the side to see where the bleb is.
9. Per rectum, the operator clears the intestines etc. away from the internal body wall at the site that the trochar will come through.
10. The helper opens the trochar/cannula package and offers the trochar/cannula to the operator.

11. Keeping one hand per rectum, with the other hand the operator places the trochar/cannula into the skin incision and pushes it HORIZONTALLY until it is through the abdominal wall, with the per-rectum hand keeping the intestines, etc. away from where the trochar/cannula will come through into the peritoneal cavity.
12. The operator then removes the trochar, leaving the cannula in place.
13. The helper then in a sterile manner picks up the needle, tubing and syringe, and offers the needle hub to the operator.
14. The operator removes the needle from the package, or the helper removes the cover from the needle tip, and the operator places the tip of the needle into the trochar and advances it to the NEAR end of the cannula (the needle does not yet stick out of the end of the cannula into the peritoneum).
15. Manipulating the ovary and the **CANNULA** per rectum, the operator places the end of the cannula firmly against the preovulatory follicle. The operator then advances the needle to puncture the follicle, and advances the needle into the follicle lumen. Follicular fluid may come into the tubing when the follicle is punctured.
16. The operator tells the helper to start pushing on the syringe. The helper depresses the contents of the syringe to inject the oocytes into the follicle.
17. When the oocyte deposit is deemed complete, the needle is withdrawn from the follicle while the helper holds and returns the system carefully to a sterile surface.
18. The oocyte delivery apparatus is returned to the lab and a 3-mL syringe with clean media is attached to the end of the tubing while the needle is held in a clean dish of media under the microscope. The line is flushed to ensure all oocytes have been deposited into the follicle and none are left behind.
19. The host mare is inseminated the same day and/or following day.
20. The mare is closely monitored to determine day and time of ovulation. This usually occurs 36-40 hours after administration of the ovulatory hormones.
21. The uterus of the host mare is flushed for embryo recovery between 7-9 days post ovulation. Four flushes are recommended. Embryos can then be transferred non-surgically to recipient mares to carry.
22. If embryos are to be vitrified, the host mare should be flushed at 7 days after transfer.

TVA approach:

1. The tail of the mare is tied to one side and the perineum is cleaned.
2. The transvaginal probe is lubricated with non-spermicidal lubricant and placed into the vagina.
3. Administer the butorphanol and buscopan to the mare.

4. The needle containing the oocytes is placed into the needle guide of the transvaginal probe.
5. Manipulating the ovary per rectum and the transvaginal probe, the operator places the end of the probe so that the dominant follicle is centered in the track that the needle will take from the probe.
6. The operator or helper then advances the needle to puncture the follicle, and advances the needle into the follicle lumen.
7. The operator tells the helper to start pushing on the syringe. The helper depresses the contents of the syringe to inject the oocytes into the follicle.
8. The air that was aspirated when the oocytes were loaded (drawn into the tubing ahead of the oocytes) can be used to mark when the oocytes should have been injected into the follicle.
9. When the oocyte deposit is deemed complete, the needle is withdrawn from the follicle.
10. The oocyte delivery apparatus is removed from the probe handle, leaving the probe handle in place.
11. A 3-mL syringe with clean media is attached to the end of the tubing while the needle is held in a clean dish of media under the microscope. The line is flushed to ensure all oocytes have been deposited into the follicle and none are left behind. If there are oocytes left behind, the apparatus can be loaded again and the follicle injection repeated.
12. The host mare is inseminated the same day and/or following day.
13. The mare is closely monitored to determine day and time of ovulation. This usually occurs 36-40 hours after administration of the ovulatory hormones.
14. The uterus of the host mare is flushed for embryo recovery between 7-9 days post ovulation. Four flushes are recommended. Embryos can then be transferred non-surgically to recipient mares to carry.
15. If embryos are to be vitrified, the host mare should be flushed at 7 days after transfer.

Laparotomy approach:

1. This approach assumes that the veterinarian is skilled in performing surgery.
2. A flank laparotomy is prepared for and performed.
 - a. For best exposure of the ovary, an incision starting just cranial to the tuber coxae and proceeding cranio-ventrally for ~ 15 cm works well.
 - b. The incision is extended into the peritoneal cavity by blunt dissection or incision of the musculature.

3. After the peritoneal cavity is reached, the ovary is identified. To locate the ovary, begin by identifying the uterine horn and following the horn to the ovary. Alternatively, sweep the hand along the muscles on the roof of the abdomen, backward into the pelvic cavity. The ovaries are suspended by a short pedicle from the dorsal body wall. See **Removal, handling and shipment of ovaries**, page 47, for related figures.
4. Bring the ovary to the incision. If good exposure of the ovary is not obtained, lengthen the incision cranio-ventrally.
5. When good exposure of the ovary is obtainable, replace the ovary back into the abdomen and have the helper load the needle apparatus in a sterile manner.
6. The helper brings the needle apparatus loaded with the oocytes to the surgeon.
7. The needle containing the oocytes is placed into the dominant follicle and the syringe is depressed to deliver the oocytes into the follicle.
8. The ovary is replaced within the abdomen.
9. The oocyte delivery apparatus is returned to the lab and a 3-mL syringe with clean media is attached to the end of the tubing while the needle is held in a clean dish of media under the microscope. The line is flushed to ensure all oocytes have been deposited into the follicle and none are left behind. If there are oocytes left behind, the apparatus can be loaded again and the follicle injection repeated.
10. When oocytes have all been delivered to the follicle, the incision is sutured closed.
11. The host mare is inseminated the same day and/or following day.
12. The mare should receive prophylactic broad-spectrum antibiotics for three days.
13. The mare is closely monitored to determine day and time of ovulation. This usually occurs 36-40 hours after administration of the ovulatory hormones.
14. The uterus of the host mare is flushed for embryo recovery between 7-9 days post ovulation. Four flushes are recommended. Embryos can then be transferred non-surgically to recipient mares to carry.
15. If embryos are to be vitrified, the host mare should be flushed at 7 days after transfer.

Expected results

1. Because this practice is still being developed and perfected, results are expected to be highly variable with little chance of success in untrained hands. This procedure should only be employed as a last attempt in extreme cases where an ICSI laboratory is not available.

Some Equine Assisted Reproductive Technology Resources

Listings are a service to readers and do not constitute a recommendation by the authors, Texas A&M University, or the Livestock Conservancy.

- Colorado State University: College of Veterinary Medicine (CO), <http://csu-cvmb.colostate.edu/academics/bms/equine-reproduction-laboratory/Pages/default.aspx> , stallion and mare services, ICSI /ART
- Equine Medical Services (MO), <http://equmed.com/reproductive-services/>, stallion and mare services, ICSI / ART
- Select Breeder Services (MD, with affiliate offices across North America), <https://www.selectbreeders.com/> , semen freezing, storage and shipping
- Superior Equine Sires (WA, including listing of freezing services), semen freezing, storage and shipping <http://www.superiorequinesires.com/veterinarians.php>
- Texas A&M University College of Veterinary Medicine & Biomedical Sciences (TX), Equine Embryo Laboratory <http://vetmed.tamu.edu/equine-embryo-laboratory> ; ICSI/ART; Theriogenology laboratory <http://vethospital.tamu.edu/large-animal-hospital/equine-theriogenology/stallion-service> , o<http://vethospital.tamu.edu/large-animal-hospital/equine-theriogenology/stallion-services>ocyte collection, semen freezing
- USDA National Animal Germplasm Laboratory, (CO), https://nrrc.ars.usda.gov/A-GRIN/main_webpage_dev/ars?record_source=US (for rare and endangered equine or livestock breeds, please contact Dr. Harvey Blackburn)
- ViaGen (TX), <http://www.viagen.com/> , cloning, somatic cell banking