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Choosing the sex of the offspring in a commercial equine embryo transfer center

JUAN CUERVO-ARANGO

Facultad de Veterinaria, Universidad CEU Cardenal Herrera, Valencia, Spain

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Cuervo-Arango J.

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Summary

It is becoming popular for horse breeders to demand to know in advance the sex of the future offspring of their mares. An example is the Polo Pony breed in Argentina, in which females are preferred over males. The main two techniques available for choosing the sex of the offspring are artificial insemination with sex-sorted semen and pre-implantation genetic diagnosis (PGD). These two techniques, however, are time consuming, expensive, and require specialized equipment and staff. Furthermore, neither technique is 100% accurate. Both of them require a confirmation of the fetal sex by transrectal ultrasonography at day 60 to day 70 of gestation. This consists in identifying the position of the genital tubercle in relation to the umbilical cord and the tail. In females, the genital tubercle is located close to the tail, whereas in males it is close to the umbilical cord, in a more cranial position. The use of this technique has become popular among field veterinarians in some countries for choosing the sex of the offspring in recipient mares after embryo transfer. This procedure involves selective abortion by the intra-uterine administration of PGF in mares carrying a fetus of the non-desired sex. Most mares expel the fetus and fetal membranes within 48 h of the treatment. Recipient mares are then used for another embryo transfer, once they resume normal cyclicity.

Keywords: sex, offspring, embryo transfer

Nowadays, it is becoming popular for horse owners to demand to know in advance the sex of the future offspring of their mares. This is especially true for breeds in which a specific sex of the horse is desired. For example, in the polo breed, females are preferred over males, since female horses are thought to perform better than males, because of their ease of training and agility. This is true to such an extent that in Argentina (the country producing the largest number of embryos of this breed and the second country in overall embryo flushings) most of polo horses born from embryo transfer are females.

There could also be other reasons for which a veterinarian may be asked to perform sex determination in a pregnant mare: curiosity, prior sales arrangements, or even the wish to test the skills of the veterinarian. The knowledge of fetal sex may be profitable for the owner, as the value of horses at sale is often determined by the sex of their offspring. Finally, the culling of broodmares is easier when fetal sex is known.

Thus, as veterinarians, we should become familiar with the available techniques for choosing and/or diagnosing the sex of the future offspring of our clients' mares. This paper focuses on reviewing different approaches for choosing and/or diagnosing the sex of the offspring with special emphasis on fetal sex determination by transrectal ultrasonography in a commercial equine embryo setting.

Semen technologies to choose the desired sex

Inseminating a mare with spermatozoa carrying the desired sex chromosome is potentially the most efficient technique to choose the sex of the future offspring. However, this technique in the equine species is not yet efficient enough – at least, not enough to be offered in a commercial setting. Flow cytometry technology was developed in 1989 (6). It consists in separating sperm on the basis of the quantity of DNA that each spermatozoon contains. Spermatozoa bearing the X chromosome have approximately 3.9% more DNA than spermatozoa bearing the Y chromosome. Since then, offspring from several different species (sheep, cow, horse, deer, etc.) bred with sex-sorted semen have been reported (3). However, it is only in the bovine species that offspring of the desired sex are obtained at a commercial level (10).

Due to the low efficiency of the sorting process (5-10 $\times 10^6$ sperm per hour), the overall dose of sex-sorted sperm obtained (between 5 and 45 $\times 10^6$ total sperm) is much lower than the standard dose used in inseminations with non-sorted sperm for mares (500-1000 $\times 10^6$

total sperm). This drawback is partly overcome by using deep-horn or hysteroscopic AI techniques close to the time of ovulation. There has been one report that describes the fertility rates in a commercial setting after deep-horn insemination of donor mares from an embryo transfer center with fresh sex-sorted semen (8). Although the overall pregnancy rate was acceptable (51.9%), the technique was time-consuming and expensive. The flow-cytometry equipment had to be close to the stallion, and the mares had to be inseminated within 12 h of ovulation, which made it necessary to perform frequent ultrasound exams. Moreover, about 10% of pregnancies were of the undesired sex. It is therefore still necessary to perform fetal sex determination by ultrasound to confirm the desired sex of the offspring.

Cryopreserved sex-sorted sperm would greatly aid the commercial development of this technique, as in the case of cattle. However, to date, the pregnancy rates for mares inseminated with frozen/thawed sex-sorted spermatozoa have been disappointingly low. In one study, the pregnancy rate after hysteroscopic insemination of mares with 5×10^6 frozen/thawed spermatozoa was 13% (7), which is unacceptable for commercial use.

Pre-implantation genetic diagnosis (PGD)

This is a promising technique to diagnose the sex of embryos flushed out of the donor mare 7 to 9 days after ovulation. This technique involves the biopsy of the embryo to recover several cells for subsequent DNA amplification by PCR. The biopsy is performed with a micropipette (4) or a Piezodrill (5), using a microscope equipped with a micromanipulator system to hold the embryo. The biopsy can be performed immediately after flushing or delayed several hours in embryos kept at a warm temperature (32°C), without affecting post-transfer pregnancy rates (4, 5). The PCR amplifies the DNA encoding the gene for the equine sex-determining region Y (*eSRY*) and amelogenin (*AMEL*). The length of the coding region for the AMEL gene is different (shorter) in the chromosome Y compared with the chromosome X. The entire PCR process takes approximately 7 h (4). Therefore the embryo should be kept at a warm temperature until the sex is known. This approach reduces markedly the number of recipient mares needed for producing the same number of foals of the desired sex, as compared with the transfer of all embryos before the sex is known (when the PCR is performed in a facility located away from the mares). For this approach, the biopsy and PCR facilities must be located at the same site. In another study involving the biopsy and PCR of embryos from a large equine embryo transfer center, out of 104 biopsied embryos, 52 (52%) had a definitive diagnosis, 17 were doubtful, and the rest could not be diagnosed (4). However, a subsequent fetal sex confirmation by ultrasound is still necessary, since there was a mismatch in 13% of embryos diagnosed as female by PCR, which proved males on ultrasound. Herrera et al. (4) developed a technique to improve the accuracy of sex determination by PCR. This consisted in incubating the sample for 10 min at 95°C before performing PCR to allow a better lysis of cells and DNA exposure. With this technique, embryo sex determination was 100% accurate.

A different approach to PGD is applied in Texas by the team of Dr. K. Hinrichs and Dr. Y. Choi (5). In this approach, embryos are shipped overnight from different places in the United States to Texas A&M Veterinary School. As soon as the embryo is received, a biopsy is taken. The samples are sent for PCR analysis to a different location. In the meantime, the embryos are either vitrified or sent back by air to the original location 4 to 6 h before transfer. Once the sex or the genetic disease status of the embryo is known, the embryo can be transferred after thawing or the pregnancy can be terminated (5). The advantage of this approach is that any veterinarian can send the embryo for genetic diagnosis, but the costs are increased because of shipping and the larger number of recipients.

Fetal sex determination by ultrasound

Fetal sex diagnosis can be performed either by transrectal or transabdominal ultrasonography. The advantages of transrectal ultrasonography are that an earlier diagnosis of fetal sex can be made (from day 59 of gestation onwards), and that no more than a standard scanner is needed (an ultrasound scanner equipped with a linear-array transducer, commonly used for routine examination of the mare's genital tract). The main disadvantage of using the transrectal approach is the increased risk of damaging the rectum (rectal lacerations), especially in nervous mares. The transabdominal approach provides for a safer diagnosis and a larger window of time during which the sex can be determined (100 to 260 days of gestation). During this wider window of time, a combination of transrectal and transabdominal techniques is often necessary. Transabdominal ultrasonography however, often requires the clipping of the abdomen and the use of a powerful scanner with a convex transducer.

Because most field veterinarians have a standard scanner equipped with a linear-array transducer, and clients of embryo transfer centers demand an early fetal sex determination, this section will focus on the transrectal approach.

There are two main anatomic regions that can be easily imaged for an accurate early sex determination: the genital tubercle (from day 59 to day 70 of gestation) and the fetal gonads (from day 90 to day 180 of gestation). A fetal sex diagnosis based on the anatomy of the fetal gonads is greatly improved by the use of Doppler technology, although it can also be determined less accurately by B-mode ultrasonography (9). For an early diagnosis, the best approach is to determine the position of the genital tubercle within the caudo-ventral region of the fetus. The genital tubercle is the precursor of the penis in the male and of the clitoris in the female. It appears as a hyperechoic bi-lobed sign located midway between the tail and the umbilical cord at day 55

of gestation. Between day 55 and day 60, the genital tubercle migrates towards the umbilical cord in the male and towards the tail in the female (2). Therefore, after day 60, a female will have the genital tubercle between the tail and the hind limbs, whereas the male will have the genital tubercle between the umbilical cord and the hind limbs. With the mare in the stocks and without sedation, a normal emptying of the rectum is performed, and the fetus is identified with a scanner. The first step is to determine the current orientation of the fetus and to confirm which side is cranial and which is caudal (the position of the head and the heart beat can be useful for confirming the orientation of the fetus within the uterus). Then, the caudal region of the fetus is imaged to find the position of the genital tubercle in relation to the umbilical cord, hind limbs, and tail.

After day 75, the genital tubercle is covered by the prepuce in the male and by the labia in the female. At this stage the identification of the prepuce or labia is harder because of the position of the fetus within the pelvic area. If the diagnosis of fetal sex is not possible at this stage, it is best to wait until day 90-100 of gestation so that the fetal gonads can be imaged (9). The fetal gonads undergo hypertrophy in the equine fetus, which greatly aids sex identification. The fetal gonads can be located near the stomach (easily imaged because of its anechoic cavity) in a dorsal position. In the male, the gonads show a homogenous echotexture with a thin central longitudinal echoic line (the testicular vein), whereas the female gonads show a central circular echoic structure surrounded by a hypoechogenic external halo. The vascularization morphology of the gonads, longitudinal (along the central line) in males and circular (around the external surface) in females, can best be imaged by Doppler ultrasonography.

Choosing the desired sex of the offspring by selective abortion

This practice has become popular in the last few years in large embryo transfer centers for polo mares in Argentina. The first reason for this practice is that there is a high demand for female offspring. Secondly, the other techniques available (AI with sex-sorted semen and PGD) are still commercially unavailable under field conditions, too costly, or unfeasible for some veterinarians.

Selective abortions, apart from being ethically controversial, have the disadvantage of requiring a large number of recipient mares, since every embryo flushed needs to be transferred into a recipient mare, and pregnancy has to be maintained until day 60 of gestation. At that point, fetal sex determination is performed by transrectal ultrasonography. Only pregnancies in mares carrying female fetuses are allowed to continue. Other pregnancies are terminated. In large embryo centers, this could mean over 500 selective abortions. When PGD or AI with sex-sorted semen are performed, there are still a number of pregnancies that have to be terminated, because of a mismatch between the PGD and the actual sex of the fetus, confirmed at later stages by ultrasonography.

A possible approach to reduce the expense of the increased number of recipient mares needed for this practice is to reuse them after abortion. However, there is the problem of eCG production by pregnant recipient mares even after abortion between day 40 and day 120 of gestation. A number of mares producing eCG may have irregular estrous cycles with formation of luteinized unruptured follicles. However, because these mares are recipients and not broodmares, they may not need to actually ovulate in order to become pregnant after embryo transfer. A recent study (1), reported a protocol to induce abortion in pregnant recipient mares between day 60 and day 70 of gestation, after diagnosing a male pregnancy by ultrasound. This technique consists in introducing an AI pipette through the cervix into the alantoic sac, rupturing the allantochorion membrane, and placing 500 μg of cloprostenol diluted in 10 mL of saline. Most mares (75%) will abort and expel the fetus and fetal membranes within 48 h of induced abortion (1). These recipient mares may be reused for another embryo transfer on average 25 days after abortion, with normal pregnancy rates of around 60%. Some mares, however, will take longer to be reusable for transfer. Some of the reasons that may explain this prolonged interval between abortion and the second transfer are that some mares may enter anestrus or have a longer period of production of luteinized unruptured follicles without showing estrus (presence of endometrial edema). In the latter case, recipient mares may be administered several treatments of PGF intramuscularly 3 to 4 days apart, until they show signs of estrus. Studies to correlate eCG production and the length of interval from abortion to re-transfer are under way.

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Corresponding author: Cuervo-Arango J., DVM, PhD, Dipl. ECAR, Facultad de Veterinaria, Universidad CEU Cardenal Herrera, Valencia, Spain