

Collection and freezing of equine epididymal spermatozoa

Afname en invriezen van epididymaal hengstensperma

K. Roels, B. Leemans, C. Ververs, J. Govaere, M. Hoogewijs, A. Van Soom

Department of Reproduction, Obstetrics and Herd Health,
Faculty of Veterinary Medicine, Ghent University
Salisburylaan 133, B-9820 Merelbeke, Belgium

kim.roels@ugent.be

ABSTRACT

The epididymis and vas deferens store an important number of fertile spermatozoa called the extragonadal sperm reserves. These stored spermatozoa can be collected in an ultimate attempt to preserve viable spermatozoa of a critically ill or dying stallion. Epididymides are collected via routine castration. After cooled transport of the testicles and epididymides, spermatozoa are collected either by retrograde flushing or by the float-up method. Retrograde flushing usually results in a much higher sperm yield and is considered the method of choice. Epididymal spermatozoa can be frozen using standard freezing protocols.

SAMENVATTING

De extragonadale spermareserves bevinden zich in de epididymis en de vas deferens. Deze spermatozoa zijn fertiel en kunnen gebruikt worden in een laatste poging om fertiele spermatozoa van een terminaal zieke of stervende hengst te bewaren. Epididymides worden verzameld via routinecastratie. Na gekoeld transport van de testikels en de epididymides worden de spermatozoa via retrograde flushing of via de flotatiemethode verzameld. Met een retrograde flushingtechniek wordt er een hogere opbrengst van spermacellen bekomen dan met de flotatiemethode. Epididymale spermatozoa worden ingevroren via standaardprotocollen.

INTRODUCTION

The first report of a foal born after artificial insemination (AI) with frozen/thawed semen, more than fifty years ago, was the result of insemination with epididymal spermatozoa (Barker en Gandier, 1957). Ever since, reports have confirmed pregnancies and/or births after AI with epididymal semen, which proves the fertilizing capacity of epididymal semen (Morris, 2002; Monteiro et al., 2011). Since billions of fertile spermatozoa can be harvested from the epididymis and ductus deferens of stallions, this sperm reservoir opens possibilities for applications in practice. For instance, when a breeding stallion requires an elective (hemi) castration or dies suddenly, a final attempt can be made to collect epididymal semen in order to preserve his genetics for future offspring. In this paper, the anatomy of the testis and epididymis, and the different steps that need to be performed in order to collect and preserve epididymal semen are

briefly described. Finally, the pregnancy results obtained with epididymal stallion semen is discussed.

THE EPIDIDYMIS: A SOURCE OF FERTILE SPERMATOZOA

The epididymis can be divided into three different parts: the head (caput), the body (corpus) and the tail (cauda) (Figure 1). Thirteen to 15 efferent ducts, originating from the testes, fuse in the head of the epididymis into one duct, i.e. the epididymal duct, which runs through the caput, corpus and tail of the epididymis. Outside the epididymis, the epididymal duct continues as the ductus deferens. As spermatozoa pass through the epididymis, they undergo maturation, and subsequently gain fertilizing ability as they enter the tail of the epididymis (Johnson et al., 1980; Amann et al., 1993). Extragonadal sperm reserves are located in the cauda epididymis as well as

in the ductus deferens, containing 62% and 7% of the total reserves of spermatozoa, respectively (Amann et al., 1978). Region-specific conditions of the tail of the epididymis keep the mature spermatozoa in a quiescent state; upon ejaculation and mixture with seminal plasma, the metabolic activity of spermatozoa increases, thus reducing their lifespan (Mann en Lutwak-Mann, 1981). A total of 15 to 25 billion spermatozoa can be collected per pair of epididymides (Bruemmer en Bruemmer, 2006; Monteiro et al., 2011; Guimarães et al., 2012). In general, both total and progressive motility are similar (Amann, 1981; Monteiro et al., 2011) or slightly higher in fresh ejaculated semen than in fresh epididymal semen (Weiss et al., 2008). However, progressive motility can be clearly diminished (Guimarães et al., 2012) or even absent (Neild, 2006) in epididymal semen. No differences are present between ejaculated and epididymal spermatozoa concerning morphological defects and viability (Weiss et al., 2008; Guimarães et al., 2012). In donkeys, a higher viability and mitochondrial activity were found in epididymal than in ejaculated semen (Gloria et al., 2011).

COLLECTION OF TESTICLES AND EPIDIDY- MIDES

Epididymides can be collected via routine castration. Halothane anesthesia has no negative effect on motility percentages and patterns (Schulman et al., 2003), but it remains unknown if local anesthesia has an effect on the quality of spermatozoa. Thus, it might be advisable to use as less local anesthetic as possible and to avoid injection into the epididymis/ductus deferens. If euthanasia is inevitable, it is preferred to perform castration prior to the injection of the drug compounds for euthanasia since their effects on semen quality and fertility is yet to be investigated (Bruemmer en Bruemmer, 2006). It is paramount that the epididymis and the vas deferens remain intact to minimize loss of spermatozoa (Bruemmer en Bruemmer, 2006). A ligature of surgical material is placed proximal on the ductus deferens during or immediately following castration to avoid sperm losses. After castration, the testicles and epididymides should be rinsed with sterile saline solution to minimize both contaminants and blood. Subsequently, the testicles and epididymides can either be processed right away or alternatively, should be placed in a passive cooling device transported for processing at a specialized facility (Bruemmer en Bruemmer, 2006). Cooling of the epididymides during transportation is a necessity to preserve the viability and fertility of the spermatozoa (Neild, 2006). It is advisable to move the epididymides as quickly as possible to a specialized center for further processing. However, it has been shown that equine epididymides can be transported for 24 hours at 5°C without a significant decrease in the fer-

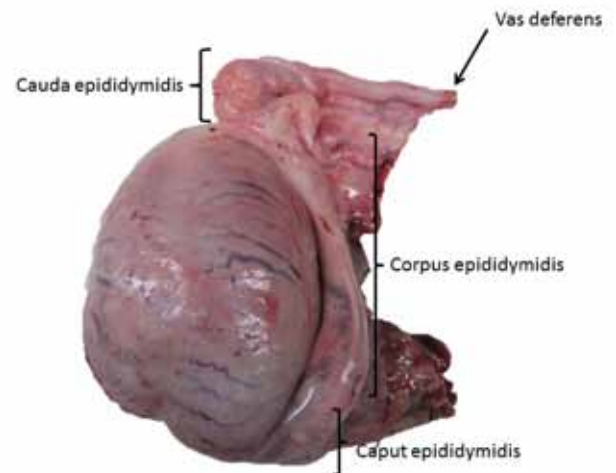


Figure 1. The different anatomic regions of the epididymis and ductus deferens.

tility of spermatozoa (James et al., 2002; Bruemmer et al., 2004; Neild, 2006; Vieira et al., 2013).

COLLECTION OF SPERMATOZOA

Upon arrival at the laboratory, the testis should be removed from the epididymis and vas deferens. Next, the stallion spermatozoa can be collected from the epididymis and vas deferens by flotation or by retrograde flushing.

The flotation method is an easy technique that does not require a lot of expertise (Cary et al., 2004; Gloria et al., 2011) (Figure 2). Twelve to 15 incisions are made along the cauda epididymis and ductus deferens before placing in a petri dish filled with approximately 5 mL of warmed semen extender. During ten minutes, spermatozoa are allowed to swim out of the epididymis and ductus deferens. Thereafter, the remnants of the epididymis and ductus deferens are removed and the extender with the semen is collected for further processing.

The retrograde flushing technique (Morris, 2002; Schulman et al., 2003; Melo et al., 2008; Heise et al., 2010; Guimarães et al., 2012) requires careful removal of the surrounding fascia of the cauda and the ductus deferens (Figure 3). Attention should be made not to cut or damage the cauda or ductus deferens. Subsequently, a syringe connected to a 14 to 18-gauge blunted needle catheter (Brinsko et al., 2003; Schulman et al., 2003), a teat cannula or a pipette tip (Melo et al., 2008) is inserted in the proximal part of the ductus deferens. An incision is made in the junction between the cauda and corpus of the epididymis. Thereafter, semen extender is passed through the cauda and ductus deferens under gentle pressure and spermatozoa can be collected in a recipient (Heise et al., 2010).

The extender used in both techniques should be



Figure 2. Flotation method.

the extender that will be used for freezing, thus containing cryoprotectant. As an alternative, the cryoprotectant can be added following the incubation period for the float-up method to reduce exposure time to the cryoprotectant.

Higher numbers of spermatozoa are found when using the retrograde flushing technique (15 to 20 x 10⁹ sperm) compared with the flotation method (4 to 5 billion sperm) (Bruemmer en Bruemmer, 2006). Due to the multiple incisions and the time allowed, there is more blood contamination when the flotation technique is used (Cary et al., 2004). However, this admixture does not seem to be disadvantageous for the results in horses (Cary et al., 2004), in contrast to what has been described in dogs (Rijsselaere et al., 2004).

FREEZING OF EPIDIDYMAL SPERMATOZOA

Freezing of epididymal spermatozoa can be performed according to standard freezing techniques (Squires et al., 1999). The ability of epididymal spermatozoa to withstand cryodamage matches the ability of ejaculated spermatozoa to withstand cryodamage within a given individual (Bruemmer et al., 2004). If semen of the stallion has been frozen earlier and the most suitable extender for this individual was determined, this extender should also be used to freeze the epididymal semen. After thawing, no differences are seen between total and progressive motility of epididymal semen compared with ejaculated semen (Weiss et al., 2008). When there are no data on the freezability of the semen of a particular stallion, BotuCrio® should be the extender of choice, because epididymal semen frozen in this extender shows a higher post-thaw total and progressive motility than epididymal semen frozen in INRA-82 or EDTA-Lactose (Melo et al., 2008). The addition of pentoxifylline in the



Figure 3. The retrograde flushing technique.

freezing extender increases both total and progressive motility; however, the effect on fertility has not been evaluated (Guasti et al., 2013). If progressive motility before freezing is absent, it is still advisable to freeze the semen, since an increase to a post-thaw motility of epididymal sperm of 35% has been recorded (Neild, 2006).

INSEMINATION WITH EPIDIDYMAL SEMEN

Pregnancies with frozen/thawed epididymal sperm have been accomplished after semen deposition in the corpus as well as in the tip of the uterine horn ipsilateral to the site of ovulation by using hysteroscopy (Morris, 2002). When the insemination dose is heavily reduced, hysteroscopic insemination favors insemination in the corpus (Morris et al., 2003; Govaere et al., 2005). Placing an insemination pipette up to the uterine horn under rectal guidance is as effective as and more time and cost saving than hysteroscopy (Brinsko et al., 2003; Govaere et al., 2014). Regardless of the insemination technique, epididymal spermatozoa should be used as efficient as possible. Seminal plasma of a stallion of proven fertility added to epididymal semen reveals contradictory results. Seminal plasma may dramatically increase the motility parameters of epididymal semen, but the beneficial effect is only partially maintained after cryopreservation (Stout et al., 2000). Neither are the effects of addition of seminal plasma on fertility rates univocal (Cary et al., 2004; Heise et al., 2010). The addition of Sperm Talp, a capacitation medium prior to insemination seems to be beneficial on pregnancy outcome when low doses of spermatozoa are used (Morris, 2002). The most sparing use of epididymal spermatozoa would be to use a single spermatozoon by intracytoplasmic sperm injection (ICSI). The type of sperm injected, i.e. frozen ejacu-

lated, cooled ejaculated or epididymal, has no influence on the pregnancy outcome after ICSI (Herrera et al., 2006; Carnevale et al., 2007). However, ICSI is a time consuming and costly technique for the mare owner, which could be a major drawback.

PREGNANCY RATES AFTER INSEMINATION OF MARES WITH EPIDIDYMAL SEMEN

Barker and Gandier (1957) were the first to inseminate with equine epididymal semen and obtained a pregnancy rate of 17%. Since then, freezing techniques have evolved and higher pregnancy rates are obtained at present. Morris et al. (2002) obtained pregnancy rates of 24% and 46% using frozen/thawed or fresh epididymal semen, respectively. A pregnancy rate higher than 65% has been noted when epididymal spermatozoa were frozen in Botucurio® (Bet labs, USA) extender (Melo et al., 2008; Papa et al., 2008).

CONCLUSION

Freezing epididymal spermatozoa is an effective and practical method of preserving a stallion's genetic potential. Since the collection of the epididymides does not require specific skills or material, it can be performed in the field by the practitioner. Further collection and processing of the semen need to be performed at an equipped center, such as the department of Reproduction, Obstetrics and Herdhealth of the Faculty of Veterinary Medicine (UGhent). Although acceptable pregnancy rates have been obtained, the efficiency of freezing and the use of frozen epididymal semen need further improvement.

REFERENCES

Amann R. (1981). A review of anatomy and physiology of the stallion. *Journal of Equine Veterinary Science* 1, 83-105.

Amann R., Hammerstedt R., Veeramachaneni D. (1993). The epididymis and sperm maturation: a perspective. *Reproduction, Fertility and Development* 5, 361-381.

Amann R., Thompson Jr D., Squires E., Pickett B. (1978). Effects of age and frequency of ejaculation on sperm production and extragonadal sperm reserves in stallions. *Journal of Reproduction and Fertility, Supplement*, 1-6.

Barker C., Gandier J. (1957). Pregnancy in a mare resulting from frozen epididymal spermatozoa. *Canadian Journal of Comparative Medicine and Veterinary Science* 21, 47.

Brinsko S.P., Rigby S.L., Lindsey A.C., Blanchard T.L., Love C.C., Varner D.D. (2003). Pregnancy rates in mares following hysteroscopic or transrectally-guided insemination with low sperm numbers at the utero-tubal papilla. *Theriogenology* 59, 1001-1009.

Bruemmer J., Bruemmer. (2006). Collection and freezing of epididymal stallion sperm. *The Veterinary clinics of*

North America. Equine Practice 22, 677-682.

Bruemmer J., Moore A., Squires E. (2004). Freezing epididymal sperm from transported testicles. *Transporting Gametes and Embryos*, 61.

Carnevale E.M., Stokes J., Squires E.L., Campos-Chillon L.F., Altermatt J., Suh T.K. (2007). Clinical use of intracytoplasmic sperm injection in horses. In: *Proceedings of the American Association of Equine Practitioners*, 560.

Cary J.A., Madill S., Farnsworth K., Hayna J.T., Duos L., Fahning M.L. (2004). A comparison of electroejaculation and epididymal sperm collection techniques in stallions. *The Canadian Veterinary Journal* 45, 35.

Gloria A., Contri A., De Amicis I., Robbe D., Carluccio A. (2011). Differences between epididymal and ejaculated sperm characteristics in donkey. *Animal Reproduction Science* 128, 117-122.

Govaere J., Hoogewijs M., De Schauwer C., De Vliegher S., Van Soom A., Duchateau L., de Kruijff A. (2014). Effect of artificial insemination protocol and dose of frozen/thawed stallion semen on pregnancy results in mares. *Reproduction in Domestic Animals* 49, 487-491.

Govaere J., Hoogewijs M., Pajmans L., Vancrombruggen I., Riga P., Sterckx J., de Kruijff A. (2005). New insemination techniques in the mare. *Vlaams Diergeneeskundig Tijdschrift* 74, 222-232.

Guasti P.N., Monteiro G.A., Maziero R.R., Martin I., Avanzi B.R., Dellaqua Jr J.A., Papa F.O. (2013). Effects of pentoxifylline on equine epididymal sperm. *Journal of Equine Veterinary Science* 33, 1153-1156.

Guimarães T., Lopes G., Ferreira P., Leal I., Rocha A. (2012). Characteristics of stallion epididymal spermatozoa at collection and effect of two refrigeration protocols on the quality of the frozen/thawed sperm cells. *Animal Reproduction Science* 136, 85-89.

Heise A., Kähn W., Volkmann D., Thompson P., Gerber D. (2010). Influence of seminal plasma on fertility of fresh and frozen-thawed stallion epididymal spermatozoa. *Animal Reproduction Science* 118, 48-53.

Herrera C., Miragaya H., Conde P., Hynes V., Losinno L., Quintans C., Pasqualini R. 2006. Intracytoplasmic injection of in vitro-matured equine oocytes with frozen-thawed epididymal sperm, 299-302. In: *Proceedings Animal Reproduction Science*. Elsevier Science bv, the Netherlands.

James A., Green H., Hoffman S., Landry A., Paccamonti D., Godke R. (2002). Preservation of equine sperm stored in the epididymis at 4 C for 24, 48, 72 and 96 hours. *Theriogenology* 58, 401-404.

Johnson L., Amann R., Pickett B. (1980). Maturation of equine epididymal spermatozoa. *American Journal of Veterinary Research* 41, 1190-1196.

Mann T., Lutwak-Mann C. (1981). *Male Reproductive Function and Semen*. Springer-Verlag, Berlin.

Melo C.M., Melo F.O., Papa E.G., Fioratti A.I.S.B., Villaverde B.R., Avanzi G., Monteiro J.A., Dellaqua D.F., Pasquini M.A., Alvarenga. (2008). Comparison of three different extenders for freezing epididymal stallion sperm. *Animal Reproduction Science* 107, 331.

Monteiro G., Papa F., Zahn F., Dellaqua Jr J., Melo C., Maziero R., Avanzi B., Alvarenga M., Guasti P. (2011). Cryopreservation and fertility of ejaculated and epididymal stallion sperm. *Animal Reproduction Science* 127, 197-201.

Morris L. (2002). The in vivo fertility of cauda epididymal

- spermatozoa in the horse. *Theriogenology* 58, 643.
- Morris L., Tiplady C., Allen W. (2003). Pregnancy rates in mares after a single fixed time hysteroscopic insemination of low numbers of frozen thawed spermatozoa onto the uterotubal junction. *Equine Veterinary Journal* 35, 197-201.
- Neild D. (2006). Cryopreservation of cauda epididymis spermatozoa from slaughterhouse testicles 24 hours after ground transportation. *Animal Reproduction Science* 94, 92-95.
- Papa F.O., Melo C., Fioratti E. (2008). Freezing of stallion epididymal sperm. *Animal Reproduction Science* 107, 293-301.
- Rijsselaere T., Van Soom A., Maes D., Verberckmoes S., de Kruif A. (2004). Effect of blood admixture on in vitro survival of chilled and frozen-thawed canine spermatozoa. *Theriogenology* 61, 1589-1602.
- Schulman M., Gerber D., Nurton J., Guthrie A., Joubert K., Volkmann D. (2003). Effects of halothane anaesthesia on the cryopreservation of epididymal spermatozoa in pony stallions. *Equine Veterinary Journal* 35, 93-95.
- Squires E., Pickett B., Graham J., Vanderwall D., McCue P., Bruemmer J. (1999). Cooled and frozen stallion semen. In: *Animal Reproduction and Biotechnology Laboratory*. Volume 9. Fort Collins (CO).
- Stout T., Morris L., Li X., Allen W. (2000). The effect of seminal plasma on the motility and cryopreservability of horse epididymal sperm. *European Equine Gamete Group (eegg)* 5.
- Vieira L., Gadea J., García-Vázquez F., Avilés-López K., Matás C. (2013). Equine spermatozoa stored in the epididymis for up to 96h at 4° C can be successfully cryopreserved and maintain their fertilization capacity. *Animal Reproduction Science* 136, 280-288.
- Weiss R.R., Weiss P.R., Muradas L.C., Graneman C., Meira. (2008). Freezing sperm from cauda epididymis of castrated stallions. *Animal Reproduction Science* 107, 356.

Erratum

In: “Antimicrobial resistance prevalence among pathogenic and commensal *Escherichia coli* from food-producing animals in Belgium” door I. Chantziaras, et al., 225-233.

Vlaams Diergeneeskundig Tijdschrift 5 - 2014, pg 232, Conclusions:

“Pathogenic *E. coli* strains both from bovines and pigs were more **pathogenic** than the respective comensal strains.”

moet zijn

“Pathogenic *E. coli* strains both from bovines and pigs were more **multi-resistant** than the respective comensal strains.”