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Introduction

Artificial insemination (AI) in horse breeding goes back to the 1930s. Pioneering work on equine AI with fresh semen has been done by Ivanow in the Soviet Union and Götze in Germany (Götze 1949). In those times the main aim of AI was to restrict disease transmission. Today AI has become a major part of breeding programs in many breeds. In European sports horse breeds such as the Hanoverian, Holsteiner, Dutch Warmblood or Selle Français AI has substantially contributed to genetic progress. Assisted reproductive technologies such as AI are now accepted by all major breed registries worldwide except the Thoroughbred race horse, although, some American breeds like the Quarter Horse have only recently started to register foals from AI with frozen-thawed semen. Many assisted reproductive technologies used today in human medicine, have been developed with substantial input from veterinary researchers. However, in contrast to their growing impact on equine breeding programmes, AI and other assisted reproductive technologies are rarely used for veterinary reasons, i.e. for increasing pregnancy rates in subfertile mares and stallions. This is not only due to costs, needs for specific equipment and low success of certain techniques but also to limited interest and experience of many equine veterinarians.

This review outlines recent developments in semen processing for AI with cooled transported or frozen-thawed semen, low-dose insemination into the tip of the uterine horn, in-vitro intracytoplasmatic sperm injection (ICSI) and separation of X and Y chromosome-bearing spermatozoa (semen sorting). The practicability of these techniques under stud farm conditions and their potential use in subfertile horses are discussed.

Semen storage and processing

A limiting factor for storage and transport of non-frozen equine semen is a rapid decrease in the fertilizing ability of spermatozoa during storage, compared to other species such as pigs and sheep. Extenders for dilution and storage of equine semen maintain adequate motility and fertility of spermatozoa for a time period of about 24 hours at 5°C. After insemination, spermatozoa of most stallions usually are able to fertilize an oocyte for up to 48 hours. A major problem are wide variations in the longevity of spermatozoa during storage between stallions and also between ejaculates in the same stallion. Extenders and storage conditions maintaining adequate semen quality for 4 to 5 days in the majority of stallions would greatly facilitate the use of cooled transported semen in the horse but such techniques are currently not available.

Most extenders for dilution, centrifugation and storage of equine semen contain either milk or egg yolk. A risk of these extenders - besides potential microbial contamination - is the fact that biological products such as milk of egg yolk consist of a variety of substances. Thus they cannot be standardized and may vary between batches. Only certain fractions may be needed for beneficial effects on sperm function and other components may even exert detrimental effects. Extenders with a defined composition containing only components with clearly protective effects on spermatozoa during storage would thus be an advantage. Fractionating of milk has alowed the preparation of purified protein fractions. Among these, phosphocaseinate and β-lactoglobulin were found to be the most effective for supporting the longevity of cooled stored spermatozoa. An extender containing these substances (INRA 96®, IMV, L’Aigle, France) was superior to milk based extenders and maintained semen quality best at a storage temperature of 15°C (Battelier et al. 1998 and 2001). However, long distance air or courier transport of semen at 15°C is not commercially available. In our laboratory, an extender containing defined components of milk only plus an extended range of sugars (EquiPro®, Minitüb, Germany) was superior to skim milk extender for semen storage also at a storage temperature of 5°C (own unpublished data).
Besides excluding undefined animal protein sources from semen extenders, the addition of antioxidants to extenders for cooled storage of semen has recently been investigated. Because of a high content of unsaturated fatty acids, sperm plasma membranes are potentially susceptible to lipid peroxidation by reactive oxygen species. Addition of the antioxidant ascorbic acid to a skim milk glucose extender increased the percentage of membrane-intact but not motile spermatozoa (Aurich et al. 1997) while addition of pyruvate increased the percentage of motile spermatozoa (Bruemmer et al. 2002) during storage at 5°C. However, in a recent study, we could show that addition of extenders without specific antioxidants markedly increases the antioxidative capacity of cooled stallion semen (Kankofer et al. 2005). Therefore addition of antioxidants to many currently used extenders may be without effect.

High concentrations of seminal plasma in cooled stallion semen are detrimental to semen motility and fertility (Jasko et al. 1992). The decrease in semen quality during cooled storage can be reduced by dilution with a suitable extender at a ratio of more than 1:2 and by removal of most of the seminal plasma by centrifugation (Aurich 2005). Although centrifugation itself can be critical for the sperm plasma membrane and fertility, especially in stallions with poor semen quality after storage, partial removal of seminal plasma by centrifugation results in a better semen quality for AI (Brinsko et al. 2000). As an alternative to partial removal of seminal plasma and for selection of highly motile spermatozoa, a variety of techniques such as swim up and filtration through glass wool, Sephadex, Percoll and Leucosorb filters have been investigated (Sieme et al. 2003). Although pregnancy rates are potentially increased when only spermatozoa selected for high motility are inseminated, these techniques are time-consuming and associated with a significant loss of spermatozoa. Further research is required before they will be used in breeding practice.

Fertility with frozen-thawed semen is lower compared to cooled semen in most mares because of reduced viability of frozen-thawed spermatozoa in the female genital tract. In addition, AI with frozen-thawed semen increases the costs for management of mares and veterinary services. Mares need to be inseminated close to ovulation which requires either repeated inseminations or examination of ovarian status at 6-hour intervals. Mares are then inseminated immediately after detection of a fresh corpus haemorrhagicum, i.e. latest 6 hours after ovulation. Alternatively, when adequate follicular development is present in an oestrous mare, ovulation can be induced with human chorionic gonadotropin and the mare inseminated 32 to 36 hours later. With adequate management and experience, per cycle pregnancy rates of 60% and seasonal rates of 80% can be obtained with frozen-thawed semen. Current research focuses on identifying critical steps during semen freezing and thawing and on optimising freezing procedures.

Low dose intrauterine insemination

For conventional AI, one insemination dose contains between 300 and 600 million motile spermatozoa. If this number could be reduced without a decrease in fertility, significantly more insemination doses could be produced from one ejaculate. In addition, a high number of spermatozoa per insemination limits the ability to exploit techniques such as semen sorting where 20 million spermatozoa or less are available per insemination. After insemination into the uterine body, only a minor part of the spermatozoa reach the uterotubal junction. If during AI semen was deposited into the tip of the uterine horn ipsilateral to the ovary bearing an ovulatory follicle, this should allow a substantial reduction in the number of spermatozoa per insemination.

In several experimental studies, mares were inseminated either deeply into the uterine horn or onto the uterine papilla (e.g. Morris et al. 2000, Lindsey et al. 2002b, Nie et al. 2003). While the first technique uses flexible pipettes which are advanced under transrectal manual or ultrasonicographic control towards the tip of the uterine horn, AI onto the uterine papilla requires visual control via a flexible endoscope (hysteroscopic AI). With semen doses between 1 and 5 million spermatozoa, pregnancy rates per insemination of 50% or more have been achieved using hysteroscopic AI. Insemination into the tip of the uterine horn with a flexible pipette requires 5 to 10 million spermatozoa (Morris et al. 2000, Lindsey et al. 2002b, Brinsko et al. 2003, Nie et al. 2003). Transrectally guided AI into the tip of the uterine horn is considerably less time-consuming and less expensive and should be considered a practical alternative to hysteroscopic AI.
Experimental results were obtained mostly under ideal conditions, i.e. with freshly collected semen, stallions selected for high fertility and induced ovulation in the mares. In most studies, highly motile spermatozoa were isolated from the ejaculate by either Percoll gradient centrifugation or other techniques before insemination. When the same experimental setup is used for conventional AI with high doses of fresh semen into the uterine body, per insemination pregnancy rates close to 90% can be reached (Buchanan et al. 2000). This indicates, that low dose insemination also under field conditions is associated with a lower pregnancy rate than conventional AI. It is the decision of the breeder to what extent this is outweighed by the potential benefits of low dose AI.

Although acceptable pregnancy rates have been obtained also with cooled (Brinsko et al. 2003) and with frozen-thawed semen (Morris 2004), fertility was reduced compared to low dose insemination with fresh semen. Pregnancy rate of subfertile stallions or stallions with low semen quality after freezing and thawing is not increased by intrauterine insemination. Simply increasing sperm numbers at the uterotubal junction by placing the inseminate near to the oviductal papilla without regarding sperm quality therefore does not improve pregnancy rates. Low dose AI with semen from highly fertile stallions might become an instrument in breeding programmes allowing to breed more mares to one stallion. However, low dose AI onto the uterine papilla is currently not a veterinary treatment to overcome male fertility problems in the horse.

**Semen sorting**

Spermatozoa bearing X and Y sex chromosomes differ in DNA content by 3.0 to 4.4%, depending on the species. Spermatozoal DNA can be stained with the bisbenzimidine stain Hoechst 33342 which binds to adenin-tymidine base pairs. Spermatozoa are then separated by fluorescence-activated cell sorting (FACS). Semen-sorting has been proposed as a tool to accelerate genetic improvement in animal breeding programmes and to control sex-linked diseases in humans. So far the technique is used commercially in cattle. Although the individual mare owner may prefer a male or a female foal, the interest of breeding programmes in sex determination of foals is limited. There may be an interest to produce more stallions than mares in the Thoroughbred race horse, however, foals conceived by technologies of assisted reproduction are not registered in this breed. A widespread use of semen sorting in other breeds is also hindered by high costs and the low capacity of current semen sorters. Maximal 25% of spermatozoa in an ejaculate are separated and the maximal capacity of semen sorters is 15 million X and Y sperm per hour.

The life-span of sorted semen in the uterus is reduced because sorting induces capacitation-like changes. Sex-sorted semen thus needs to be combined with low dose insemination close to ovulation (Seidel and Garner 2002, Maxwell et al. 2004). If this is taken into account, per insemination pregnancy rates between 30 and 50% can be met with fresh, sorted semen while pregnancy rates are reduced with frozen-thawed, sorted semen (Buchanan et al. 2000, Lindsey et al. 2001 and 2002a).

Commercial equine AI centres currently usually cannot afford semen sorting equipment and, under field conditions, stallions in most cases will not be sent to the location of a semen sorter. However, semen has been stored for up to 18 hours at either 5 or 15°C before sorting. Under experimental conditions, a pregnancy rate of 72% was obtained when this semen was used for hysteroscopic insemination (Lindsey et al. 2005). Shipment of semen from the collection centre to the sorting institution is thus possible without a major decrease in fertility.

**IVF and ICSI**

In vivo reproductive biotechnologies depend on the availability of equine oocytes. While research institutions can use oocytes obtained from slaughterhouse material, the only technique available for recovery of oocytes from individual mares is ultrasound-guided puncture of pre-ovulatory follicles through the vaginal wall and aspiration of oocytes. On average 3 to 5 oocytes per mare can be obtained in one session (Kanitz et al. 2000).

In vitro fertilization of oocytes, a technique used in human assisted reproduction and for production of cattle embryos, has found no entry into horse breeding. Only two foals have been born in research institutions following IVF of in vivo matured oocytes recovered by trans-vaginal ultrasound-guided pick-up (Palmer et al. 1991). Success of this technique in the horse has been hindered by the reduced

The restrictions of IVF have been largely overcome by the development of the technique of intracytoplasmatic sperm injection (ICSI). ICSI requires a mature oocyte in metaphase II and fresh or frozen-thawed spermatozoa. The oocyte can be matured either in vivo in a pre-ovulatory follicle after GnRH stimulation or in vitro. With a micromanipulator, a single spermatozoon is injected directly into the cytoplasm of the oocyte. The use of a Píezo drill facilitates penetration of the zona pellucida and ensures breakage of the sperm and oocyte plasma membranes (Hinrichs 2005). Advantages of the method include the fact that spermatozoa can be immotile and that ICSI overcomes fertilisation problems caused by defect spermatozoa. The zygote created undergoes several divisions to the morula or early blastocyst stage before being transferred transcervically to a synchronised recipient mare (Cochran et al. 1998). Under optimal conditions, 80% of the zygotes start mitotic division but less than 35% progress to the blastocyst stage. Out of the transferred blastocysts, 50% of result in a pregnancy and about half of these pregnancies are carried to term (Hinrichs 2005). In order to increase pregnancy rates after transfer of ICSI-derived embryos, either early embryonic stages need to be transferred directly into the oviduct of recipient mares or methods for embryo culture need to be improved.

In humans, spermatozoa with extensive damage of the nuclear DNA can lead to pregnancies following ICSI (Twigg et al. 1998). The risks inherent in using such assisted conception techniques in males with fertility problems are apparent. A significantly elevated incidence of major birth defects is seen in children conceived by ICSI (Kurinczuk and Bower 1997), although, this incidence is not greater than that in children generated by IVF (Hansen et al. 2002). The impacts of assisted reproductive technologies on birth defects in foals currently remain unclear but should be lower when such techniques are used with selected fertile stallions as part of breeding programme compared to their use in horses with fertility problems.

References


