Pferdeheilkunde 19 (2003) 6 (November/Dezember) 675-676

Sexing of Stallion Semen

Detlef Rath and Harald Sieme[1]

Institute of Animal Science, Mariensee (FAL) and National Stud, Celle[1]

Summary

Separation of X-and Y-chromosome bearing spermatozoa to pre-select the sex of offspring is now possible in most large farm animals including horses. Spermatozoa are separated by flowcytometry based on the amount of DNA that differs between the both sex chromosomes. The purity of the sex selection is very high, however the number of spermatozoa being separated is limited. Therefore AI strategies have to be modified in order to get normal pregnancy rates even with low dose insemination. Recently it was shown that hysteroscopic insemination into the tip of the uterus horn or at the uterine papilla results in acceptable pregnancy rates. This review summarizes the data being provided in literature when both technologies, sperm sexing and low does insemination, are combined, employing freshly collected sorted semen, fresh semen stored for 18h prior to sorting and sorted, frozen/thawed spermatozoa. Several foals have been born so far, but the technology needs further improvement before being advertised for commercial use.

Keywords: Sperm sexing, Flowcytometry, Low dose insemination; Hysteroscopic insemination

„Sperm-sexing" beim Hengst

Die Trennung von Samenzellen entsprechend ihrer Geschlechtschromosomen, um Nachkommen mit vorausbestimmten Geschlecht zu produzieren, ist für die meisten landwirtschaftlichen Nutztiere incl. Pferd möglich. Die Samenzellen werden aufgrund des unterschiedlichen DNA-Gehaltes der Geschlechtschromosomen flowzytometrisch getrennt. Die Trenngenauigkeit ist sehr hoch, allerdings ist die Zahl an Samenzellen, die pro Zeiteinheit getrennt werden können, limitiert. Daher müssen die Verfahren zur Besamung der Stuten entsprechend angepasst werden. Kürzlich wurde gezeigt, dass eine intrauterine Besamung in das Uterushorn bzw. auf die Papille mittels Hysteroskopie zu vertretbaren Trächtigkeitsraten führt. Dieser Übersichtsartikel fasst die bislang veröffentlichten Daten zusammen, soweit sie sich auf die Besamung mit gesextem Sperma und hysteroskopischer Besamung beziehen. Dazu wurde Sperma direkt nach der Gewinnung sortiert, nach der Gewinnung und vor dem Sortiervorgang 18h gelagert oder nach dem Sortieren tiefgefroren. Mehrere Fohlen sind mittlerweile geboren. Die Techniken müssen aber deutlich verbessert werden, bevor ein Einsatz für die Pferdezucht sinnvoll wird.

Schlüsselwörter: Sperma-Sexing, Flowzytometrie, Besamung mit geringer Spermienzahl; Hysteroskopische Besamung

Review

Improving the reproductive efficiency of horses is dependent on the development of new biotechnologies that can be effectively applied to industry. Two such technologies are gender pre-selection and low dose insemination. The only proven sexing procedure is the Beltsville Sperm Sexing Technology (*Johnson* et al. 1989). This method has consistently produced offspring of the predicted sex in many farm animals. Six years ago the sorting technology was improved tremendously (*Johnson* et al. 1989, *Johnson* 1997). The introduction of high speed flowcytometry allows sorting of up to 15 million spermatozoa per hour based on the DNA content of the heterosomes. The relative DNA difference between X-and Y-chromosome bearing spermatozoa is species specific and reaches 4.2% in equine spermatozoa. This small difference is enough to distinguish between the two sperm populations.

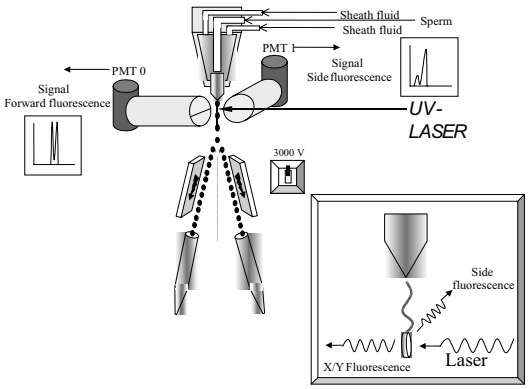
For sperm sorting, individual spermatozoa are labeled with a fluorescence dye (Hoechst 33342). The fluorescence signals of the stained spermatozoa are detected by exposure to UV-Laser light in a flowcytometer (UV Argon Laser, 150 mW). About 25.000 to 30.000 droplets can be recognized per second, out of which up to 5.000 spermatozoa are selected per second per sex with high sorting purity. Reanalysis of purity is performed directly after sorting and is usually higher than 90%.

Meanwhile several trials have shown the production of foals with sex selected spermatozoa. *Schmid* et al. (2000) reported about the first foal after surgical insemination with 150.000 spermatozoa. The first successful non surgical insemination was performed in a study by *Buchanan* et al. (2000). Mares were inseminated once with 25 million motile spermatozoa, diluted in 1 ml of a skim milk extender. The semen was deposited into the tip of the uterine horn ipsi-lateral to the pre-ovulatory follicle. At 16 days after AI, pregnancy rates were 30% but had decreased to 10% by day 60. A second group of mares was inseminated in the same way, except that 4% of egg yolk were added to the skim milk extender. Pregnancy rates with sex ratios of offspring from 87% to 89% of the desired sex were 50% on day 16 after AI and 40% on day 60. As mares are normally inseminated with 500 million progressively motile fresh spermatozoa or about 1 million spermatozoa that have been frozen and thawed these results with extremely low sperm numbers are very noteworthy .

As spermatozoa are identified individually by flowcytometry their availability is limited. In consequence, either in vitro production techniques or modified AI protocols need to be used to produce offspring. As the survival time of sorted equine spermatozoa is very limited, AI needs to be performed as soon as possible after sorting and preferably at or shortly after ovulation. As in other species, location of semen deposition, amount of spermatozoa used per AI and timing of insemination differ from conventional AI. A valuable method to deposit the semen closer to the site of fertilization is the video-endoscopically guided hysteroscopic insemination (Morris et al., 2000, 2003). In a recent study Lindsey et al. (2002) showed that a pregnancy rate of 25% can be obtained with sorted

semen employing this technology. In an earlier trial Lindsey et al. (2000) investigated if semen can be stored for 18h prior to sexing. Although the number of replicates was low, pregnancy rate after AI with pre-stored semen were slightly better (35%) as compared to samples sorted directly after semen collection (30%).
In addition to semen immediately sexed after collection, fresh stored for 18h and then sexed or unsorted semen, Lindsey et al. (2001 and 2002) used sexed frozen/thawed semen at low concentrations for AI in 2 subsequent trials. Table 1 summarizes the data. High pregnancy rates were obtained with pre-stored sexed semen when 20 million spermatozoa were inse-

Fig 1 Principles of sex separation of spermatozoa by flowcytometry
Prinzip des „Sperma-Sexing" mittels Flowzytometrie



minated hysteroscopically. Insemination results employing 5 million spermatozoa did not differ between fresh sorted and unsorted frozen semen, whereas sexed frozen/thawed samples resulted only in 13% of the mares becoming pregnant. Freezing of sorted semen needs further improvements before recommended for commercial AI.
In summary, as in other species sperm sorting technology has become reality for equine AI and can be used with fresh sor-

Fig 2 MoFlo® Dakocytomation Flowcytometer, modified for sperm sorting
MoFlo® Dakocytomation Flowzytometer, modifiziert zur Spermientrennung

ted semen or even after storage 18h before sorting, provided insemination is made by hysteroscopy. Even sorted frozen samples can be used, however to make them available for breeding purposes its quality needs further improvement. In an experiment starting this year in cooperation between the Institute of Animal Science in Mariensee and the National Stud in Celle, freshly sorted and sorted frozen semen will be

Tab 1 Hysteroscopic and non surgical deep intrauterine insemination of sex-sorted and non sorted spermatozoa using different concentration of fresh or stored or frozen/thawed semen (mod. from *Lindsey* et al. 2001 and 2002)
Hysteroskopische und nicht chirurgische intra-uterine Besamung mit gesextem und ungesextem Spermien bei Verwendung verschiedener konzentrationen von frischem und gelagertem gesextem Sperma sowie tiefgefrorenem/aufgetautem Sperma (mod. nach Lindsey *et al. 2001 und 2002)*

Insemination technique	No. of spermatozoa ($x10^6$)	Type of spermatozoa	No. of mares / group	% Mares pregnant
Hysteroscopic	0.5	Fresh/sorted	15	33
insemination	5	Fresh/sorted	15	37,5
	5	Stored 18h/sorted	20	25
	5	Sorted/Frozen/thawed	15	13
	20	Stored 18h/sorted	14	72
	5	Fresh/**non**-sorted	10	40
	5	Frozen/thawed/**non** sorted	16	37.5
Deep	5	Fresh/sorted	10	0
intrauterine	25	Stored 18h/sorted	20	40
insemination				

used for selected inseminations, comparing different semen preparations and insemination techniques. Finally, it has to be mentioned that the sperm sexing technology is patented and a license is required for commercial use by XY Inc, CO, USA.

Literature

Buchanan B. R., Seidel Jr. G. E., McCue P. M., Schenk J. L., Herickhoff L. A. and *Squires E. L.* (2000): Insemination of mares with low numbers of either unsexed or sexed spermatozoa. Theriogenology 53, 1333-1344

Johnson L. A., Flook J. P. and *Hawk H. W.* (1989): Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod 41, 199-203

Johnson L. A. (1997): Advances in gender preselection in swine. J Reprod Fertil Suppl 52, 255-266

Lindsey A. C. (2000): Hysteroscopic insemination with low numbers of fresh and cryopreserved flow sorted stallion spermatozoa. M.S. Thesis, Colorado State University, Fort Collins, CO, USA

Lindsey A. C., Bruemmer J. E. and *Squires E. L.* (2001) : Low dose insemination of mares using non sorted and sex-sorted sperm. Anim Reprod Sci 68, 279-289

Lindsey A. C., Morris L. H., Allen W. R., Schenk J. L., Squires E. L. and *Bruemmer J. E.* (2002): Hysteroscopic insemination of mares with low numbers of nonsorted or flow sorted spermatozoa. Equine Vet J 34, 128-132

Morris L. H., Hunter R. H. F. and *Allen W. R.* (2000): Hysteroscopic insemination of small numbers of spermatozoa at the uterotubal junction of preovulatory mares. J Reprod Fertil 188, 95–10

Morris L. H., Tiplady C. and *Allen W. R.* (2003): Pregnancy rates in mares after a single fixed time hysteroscopic insemination of low numbers of frozen-thawed spermatozoa onto the uterotubal junction. Equine Vet J 35,197-201

Schmid R. L., Kato H., Herickhoff L., Schenk J. L., McCue P. M., Chung Y. G. and *Squires E. L.* (2000): Effects of follicular fluid or progesterone on in vitro maturation of equine oocytes before intracytoplasmic sperm injection with non-sorted and sex-sorted spermatozoa. J Reprod Fert 56, 519–525

Prof. Dr. Detlef Rath
Inst. of Animal Science, Federal Agricultural Research Center (FAL)
31535 Neustadt
rath@tzv.fal.de