

In Vitro Evaluation of Frozen-Thawed Stallion Semen: A Review

By T. Katila

Department of Clinical Veterinary Sciences, University of Helsinki, Finland.

Katila, T.: *In vitro* evaluation of frozen-thawed stallion semen. A review. Acta vet. scand. 2000, 42, 201-217. – The article reviews methods used for in vitro evaluation of sperm, with particular emphasis on frozen-thawed stallion sperm. The techniques, limitations of the methods and correlations with fertility results are discussed. Very few studies have tried to find correlation between fertility of frozen stallion semen and laboratory tests. It is difficult and expensive to inseminate an adequate number of mares to achieve statistically significant differences. Significant, but low correlations have been demonstrated between the foaling rate and subjective motility of sperm incubated for 2 h and 4 h at 37°C and hypoosmotic swelling test after 0 and 3 h of incubation. Significant correlations have been reported between the pregnancy rate and viability of propidium iodide-stained sperm assessed by flow cytometry as well as for glass wool and Sephadex filtration tests. No correlations have been detected between fertility and motility immediately after thawing. In spite of that, motility estimation by light microscope is the most commonly used method to evaluate frozen-thawed stallion sperm. Computer assisted automatic sperm analyzers have replaced light microscopy in research projects, but so far nobody has been able to demonstrate a correlation between fertility of frozen stallion semen and any of the motility parameters obtained by these instruments.

horse; sperm; cryopreservation; semen quality; motility; membrane integrity.

Introduction

There is considerable variation between individual stallions in how well their semen retains its fertilizing capacity after freezing and thawing. It has been estimated that only 20% of fertile stallions produce sperm that survive well the freezing and thawing processes (Tischner 1979). Although our knowledge and techniques have improved within the last 20 years, a considerable proportion of stallions are still not suitable for semen freezing. About 50% of examined French stallions showed poor freezability of sperm (Vidament *et al.* 1997). However, according to Mennick (1997), stallions which have passed the breeding health examination hardly ever are truly "poor freezers". It is only a matter of finding suitable freezing extenders

and methods for individual stallions (Mennick 1997, Loomis 1999).

Development of freezing methods requires in vitro tests that correlate with in vivo fertility, but controlled breeding trials with an adequate number of horses are extremely expensive (Loomis 1999). Amann (1989) gives a good example: if we inseminate 10 mares, with the 95% confidence interval for the "true fertility" of 50%, the stallion's "observed fertility" would be between 15% and 85%! Similarly, assuming a "true fertility" of 50%, the 95% confidence interval for the "observed fertility" based on 100 inseminations is 40% to 60%, and 47% to 53% if based on 1000 inseminations. It is hard to imagine that we could have hundreds of mares in frozen semen insemination trials.

With the increasing international trade and commercial use of frozen semen, the unacceptably poor pregnancy rates cause considerable frustration and economic losses in the equine breeding industry (Boyle 1996). The slow progress in the development of freezing techniques for equine semen is partly explained by the lack of reliable laboratory methods. Some in vitro methods work reasonably well in the assessment of fresh semen, the best example being motility evaluation. In spite of its limited applicability, motility is the most commonly used parameter in the evaluation of frozen-thawed semen, in both laboratories and stud-farms, because it is easily accessible and quick to perform. It is generally agreed that tests other than in vitro motility could be important for predicting fertility. Numerous promising assays have been reported in the literature but few have found their way into commercial semen freezing laboratories (Loomis 1999). A combination of laboratory tests should enable better assessment of the fertility potential of cryopreserved stallion semen (Blach *et al.* 1989).

Motility

Sperm motility is important because it is readily identifiable and reflects several essential aspects of sperm metabolism. Therefore, motility should be evaluated together with other parameters when estimating the fertilizing potential of spermatozoa. Usually total motility (any type of motility) and progressive motility (spermatozoa moving actively forward) are estimated as percentages. Motility can also be described as circling, oscillating and serpentine (Kenney *et al.* 1983). Often also the speed of spermatozoal motion is assessed. If semen is exposed to low temperatures or it dries on the slide, motility diminishes rapidly.

Stallion spermatozoa have some species-specific characteristics: an asymmetrical head, an abaxial position of the tail, an acrosome of

small volume and the presence of microtubules in the neck (Bielanski & Kaczmarek 1979). The large, circular motion of normal sperm is due to a high incidence of abaxial connections between the sperm head and neck (Kenney *et al.* 1983). Estimating only the progressive motility may underestimate good motility of some stallions.

Light microscopy

To obtain an accurate estimate, environmental conditions should be standardized and optimal for semen. All equipment should be clean (preferably disposable) and before use, kept at body temperature by storing in an incubator. If the semen sample is too thick, spermatozoa are in layers and motility cannot be reliably estimated. Samples of a higher concentration are usually judged by the human eye as having higher motility (Jasko 1992). Semen should be extended to $(25 \text{ to } 50) \times 10^6$ spermatozoa/ml, but not with a diluent that influences motility. Temperature of the slide should be controlled ($+37^\circ\text{C}$) by using a stage warmer on a phase-contrast microscope, the depth of suspension on the slide should be standardized and multiple fields near the centre of the slide examined. Motility at the edges declines more rapidly than in the centre as a result of drying and exposure to air. (Jasko 1992). The light microscopic evaluation does not require expensive equipment and is easy to perform. However, the greatest variation is caused by a variation between examiners, since the evaluation is subjective and requires experience.

When fresh stallion semen was subjectively evaluated, low correlations were found between fertility and the percentage of motile ($r = 0.40$) and progressively motile ($r = 0.46$) spermatozoa (Jasko *et al.* 1992). The number of mares inseminated with frozen semen has, in most experiments, been so small that statistical evaluation of the data has not been feasible. This may

be one reason why very little published data exist on the correlation of motility evaluated by light microscopy and fertility of frozen-thawed stallion semen. In a study where 177 mares (on average 19 mares/stallion; min 6, max 51) were inseminated with frozen semen from 9 stallions, the correlation coefficient of the visually estimated percentage of motile cells to the first-cycle pregnancy rate was only 0.32 (*Samper et al.* 1991). Good motility of frozen-thawed semen was a poor indicator for pregnancy rates in pigs (*Hammitt et al.* 1989). Similarly, in the horse, the percentage of progressively motile, post-thaw spermatozoa is considered to be a poor predictor of pregnancy rates in mares (*Pickett et al.* 1987, *Squires et al.* 1987, *Bataille et al.* 1990, *Wilhelm et al.* 1996). Female genital fluids exert an influence on sperm motility. Some sperm that are immotile in vitro might regain motility in vivo, and vice versa (*Blach et al.* 1989). A very low motility would probably be an indication not to use the semen, but a good motility does not necessarily indicate that the fertilizing capacity of spermatozoa has been maintained.

Computer-aided sperm analysis (CASA)

Subjective visual evaluation of motility is prone to human error and bias. Therefore, objective methods have been developed. Methods based on microscopic images include time-lapse photomicrography (*van Huffel et al.* 1985), multiple-exposure photomicrography, frame-by-frame playback videomicrography and cinematography (*Tischner* 1979), whereas turbidimetry, spectrophotometry and laser Doppler technology are based on physical principles (*Comhaire et al.* 1992). Because photographs are tedious to analyse, computer-assisted technologies were the next step in the development of automated motility analysis. Due to the high cost of the instrument, computerized sperm image analysis systems are used

primarily for research applications. The first systems available were the CellSoft Automated Semen Analyser and the Hamilton Thorn Motility Analyzer (HTM), others have since been introduced to the market.

Video images for computerized sperm motion analysis are obtained from viewing fields of motile sperm using a microscope. A set number (usually 20 to 30) of successive video frames is analysed at a constant rate, typically 30-60 frames per second. When all frames for a given field have been analysed, computer algorithms are used to distinguish sperm from non-sperm objects and to reconstruct sperm tracks (*Jasko* 1992). Each sperm is classified as either motile or nonmotile, and the concentration of both is calculated. Motility data is further characterized as follows: mean curvilinear velocity (VCL), path velocity (VAP), mean straight-line velocity (VSL), straightness (STR = VSL/VAP), linearity (LIN = VSL/VCL), percentages of total motility (MOT), progressive motility (PMOT), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). The newest models also provide morphological measurements for certain species, although limited to sperm head morphology. Automated morphology systems have been validated for human sperm but not for horse. What all these specific motility characteristics tell us about the quality of fresh or frozen stallion semen is somewhat unclear because standard values have not been defined for normal or abnormal sperm motion. No international standardization in equipment settings has yet been implemented. The selection of gates, minimum and maximum values for head size and brightness, minimal velocities, straightness, frame rate, etc. influence results accordingly, and thus, do not allow comparison of results between laboratories. There is an urgent need for users of CASA to agree on standard analysis parameters within a given species.

In the analysis of frozen semen, particularly, non-spermatozoal particles (e.g. egg yolk) can mistakenly be identified as spermatozoa, causing "background noise". As a result, not only will the sperm concentration be overestimated, but the proportion of motile spermatozoa will be miscalculated (Comhaire *et al.* 1992). The effect of egg yolk particles on many motion characteristics has been shown by Ziegler (1991). If thawed semen is greatly diluted with a clear extender, the number of egg yolk particles and the concentration of viscous glycerol decrease. Varner *et al.* (1991a) used nonfat dry milk-glucose extender to dilute frozen-thawed semen samples before CASA evaluation. One approach to analysing frozen semen is to use clarified freezing extender which is prepared by centrifuging egg yolk with extender at $10\,000 \times g$ for 15 min. The supernatant including the lipid on the surface is then mixed with the freezing extender (Burns & Reasner 1995). Filtering of extender through a $0.2\text{-}\mu\text{m}$ membrane filter removes larger particles that could interfere with measurements (Budworth *et al.* 1988). Recently, fluorescence dyes that do not affect motility (Hoechst 33342) have been used to differentiate sperm cells from egg yolk particles in CASA systems equipped with the epifluorescent illumination (Hamilton Thorne IVOS) (Farrell *et al.* 1996).

The maximum sperm concentration in CASA-systems is usually $50 \times 10^6/\text{ml}$. A dilution of stallion semen to $25 \times 10^6/\text{ml}$ has been recommended (Varner *et al.* 1991a). Varner *et al.* (1991a) used Makler-chambers and videotaped the semen samples. This considerably shortens the time that semen samples have to stand in the Makler-chamber as compared to performing the analyses right away. Sperm dries quickly in a Makler-chamber at 37°C which is a problem in the older, slower, analyzers, but the newest CASA-instruments are able to analyse 400 cells in 2 min. In the study of Varner *et al.* (1991a),

the most highly variable component was field within chamber. They recommended that 3 chambers/ejaculate and 3 fields/chamber be evaluated which would yield a mean spermatozoal number of approximately 500 evaluated per sample.

No significant correlations were found when ca 20 000 cows were inseminated with frozen semen from 10 bulls and the 75-day nonreturn rate was compared with motility characteristics obtained by CellSoft Analyser (Budworth *et al.* 1988). In another fertility trial, the competitive fertility index for 9 bulls was correlated ($r > 0.68$) with MOT, VCL and VSL (Budworth *et al.* 1988). MOT had a low (0.45) but significant correlation with the first-cycle pregnancy rate of 177 mares inseminated with frozen semen from 9 stallions (Samper *et al.* 1991). In a French study, in which 60 ejaculates were frozen from 7 stallions, batches with a post-thaw motility of $>35\%$ accepted for use, and 334 mares inseminated, there was no correlation between fertility and subjective post-thaw motility or percentage of sperm moving $>30 \mu\text{m}/\text{sec}$ (RAP) analysed by CASA (Bataille *et al.* 1990). In another French study, 766 mares were inseminated with frozen semen, but none of the criteria measured by CASA (VCL, LIN, ALH, MOT, RAP) had a significant correlation with fertility (Palmer & Magistrini 1992).

It is not surprising that CASA-systems have been unable to detect differences between "good" and "poor" frozen semen when the ejaculates have been selected to include only those with a post-thaw progressive motility $>30\%$ to 35% . The fertility of mares varies widely and one has to bear in mind that a single ejaculate can only be used for 5 to 15 mares. If low-quality semen had not been rejected before freezing, CASA would probably have detected significant differences more readily. On the other hand, computers are not needed to detect large differences. The much less expensive way of

subjectively evaluating total or progressive motility using a light microscope yields similar results to automatic analysers (*Samper et al.* 1991, *Palmer & Magistrini* 1992, *Kneissl* 1993). To date, the superiority of the automatic analyser in the evaluation of frozen semen has not been proven, although it is the only way to accurately assess velocity and linearity. It should be emphasized that automated analysis presents risks of artifacts that must be controlled for and that the apparatus must be correctly set (*Palmer & Magistrini* 1992). It is worrying that the mean motility values obtained from the same semen samples sometimes differed by as much as 30% when analysed simultaneously by CellSoft and HTM (*Jasko et al.* 1990b). Further, when the same semen specimens were analysed by 2 identical HTM analysers, significant differences were seen in sperm counts, ALH, LIN and BCF, which shows that the reproducibility was poor (*Agarwal et al.* 1992).

Longevity of motility (survival tests)

For estimating the longevity of motility, an aliquot of well-mixed – typically extended – semen is used to fill a warm sterile tube which is kept in a draft-free, preferably dark environment. The semen is mixed and an aliquot examined at regular time intervals until <10% of the sperm remain progressively motile (*Kenney et al.* 1983).

The incubation temperatures and times have varied considerably. Longevity of motility increases with decreasing temperature. *Müller* (1982, 1987) used survival for >120 h at 2–4°C or at 1–4°C as a criterion for accepting frozen semen for field use. The average time for accepted semen was 202 hours, with a range from 120 to 312 h. Survival tests are in routine use in some stallion stations: 37°C for 4 h (threshold motility 15%), 20°C for 12 to 48 h (threshold 5–10%) and 5°C for 7 days (threshold 5%) (*Vida-*

ment et al. 1998). Other laboratories employ shorter incubation times at 37°C, e.g., only 0.5 h (*Loomis* 1999). In a retrospective study on commercially used frozen semen with 31 stallions and 1023 mares the thawed semen was kept at 37°C. A significant correlation was demonstrated between the foaling rate and motility evaluated by light microscopy after an incubation of 2 and 4 h (*Katila et al.* 2000a).

Morphology and membrane integrity

In some studies increases in sperm abnormalities have been associated with decreased fertility (*Bielanski* 1975, *Jasko et al.* 1990a), but others have found no relation between morphology of fresh semen and fertility (*Voss et al.* 1981, *Dowsett & Pattie* 1982). A wide range of morphological deviations may be acceptable for breeding stallions, if the total number of morphologically normal motile spermatozoa in the ejaculate is adequate (*Kenney et al.* 1983). Sometimes the low pregnancy rates after frozen semen inseminations are simply due to an excessively small number of live morphologically normal post-thaw sperm. Morphological features are evaluated by light microscope using different sperm stains. The use of fluorescent probes requires epifluorescence optics for the microscope. Scanning and transmission electron microscopic techniques are not in routine use, but have been useful in some abnormal cases and in research. One has to be cautious in the interpretation of transmission images. *Abraham-Peskir et al.* (2000) noticed that membrane-bound vesicles in acrosomal and midpiece regions are not caused by freezing and thawing. They are damaged during preparation of samples.

After freezing and thawing, ultrastructural changes were observed in the acrosome, in the outer fibres of the midpiece, and in the axoneme of the principal piece (*Christensen et al.* 1995). Automated morphometric analysis provides ac-

curate objective measurements of sperm head and shape (Davis *et al.* 1993, Magistrini *et al.* 1997).

Conventional stains

The simplest examination method is to fix sperm cells in buffered formal-saline or buffered glutaraldehyde solution and view unstained cells with either phase-contrast or differential interference-contrast microscopy. General-purpose cellular stains (Wright's, Giemsa, haematoxylin-eosin, India ink) can be used (Varner *et al.* 1991b), but live-dead stains (aniline-eosin, eosin-nigrosin, eosin-fast green) are more widely used for the determination of cell viability. Integrity of the plasma membrane is shown by the ability of a viable cell to exclude the dye, whereas the dye will diffuse passively into sperm cells with damaged plasma membranes (Colenbrander *et al.* 1992). Glycerol can interfere with the staining properties of these dyes making them less reliable for the evaluation of cryopreserved semen (Wilhelm *et al.* 1996). Differential stains for sperm cells are Spermac (Oettle 1986), William's and Casarett's stains (Kenney *et al.* 1983), Triple stain, Papanicolau, and Feulgen and Karras among others (Magistrini *et al.* 1997). The Spermac stain was not found to be very useful in the evaluation of frozen stallion semen by Wöckener and Schuberth (1993), although it has been in routine use in Germany (Schrop 1992). It is generally recommended that 200 cells be examined, but evaluation of 100 sperm cells probably provides a valid representation of abnormalities (Hermenet *et al.* 1993).

Fluorescent stains

A combination of 2 fluorescent stains, e.g. carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) or calcein AM and ethidium homodimer, can be used to assess cell viability. CFDA and calcein AM molecules cross cell

membranes and are de-esterified by esterases within the cell. They are retained within intact cells, causing them to fluoresce green. PI and ethidium homodimer cannot penetrate living cells, but can only bind to and stain cellular DNA in damaged cells, giving them red fluorescence (Malmgren 1997). Other frequently used fluorescent dyes are Hoechst 33258, ethidium bromide (EB) and SYBR14.

The most commonly used method to detect acrosome integrity is staining with fluorescein-conjugated lectins, such as Peanut Agglutinin (PNA), Pisum Sativum Agglutinin (PSA) or Concanavalin A (ConA) coupled with fluorescein isothiocyanate (FITC) (Magistrini *et al.* 1997, Blanc *et al.* 1991). FITC-PNA with ethidium homodimer as a counter stain allowed for a rapid and reliable assessment of the acrosomal status of stallion sperm. Acrosome-intact spermatozoa displayed intense green fluorescence over the acrosomal cap, while acrosome-reacting spermatozoa showed a patchy disrupted image of fluorescence. Sperm cells that had completed the acrosome reaction acquired a stain on the equatorial segment or remained unstained (Cheng *et al.* 1996). Chlortetracycline assay (CTC) is used to detect capacitation and acrosome reactions of the spermatozoa (Varner *et al.* 1993). Mitochondrial activity can be evaluated by Rhodamine 123 (R123), which is a fluorescent dye used to label a negative potential (the inside of the mitochondria being negative) across the inner mitochondrial membrane. Only coupled, respiring mitochondria will take up this fluorescent dye. A good correlation has been shown between sperm viability and mitochondrial function for equine spermatozoa (Casey *et al.* 1993, Papaioannou *et al.* 1997). Gravance *et al.* (2000) used another fluorescent dye, JC-1, to assess mitochondrial function in equine sperm. They concluded that JC-1 accurately reflects changes in mitochondrial membrane potential.

Typically, 100 to 400 fluorescent cells are counted under microscope. A fluorometer can be used to evaluate the proportion of fluorescent cells rapidly. This method has been applied to frozen boar sperm (Eriksson *et al.* 1998) and also to fresh (Gravance *et al.* 2000) and frozen stallion semen (Katila *et al.* 2000a and b). In our study, frozen-thawed stallion sperm were stained with PI and fluorescence determined; however, no correlation with fertility was established (Katila *et al.* 2000a and b). A very rapid and effective method is flow cytometry, which allows thousands of individual cells to be evaluated. Multiple aspects of sperm function can be assayed simultaneously. Sperm viability, DNA content and the proportion of acrosome-reacted sperm can be investigated using this method (Morrell 1991). The cost of sorting flow cytometry at the moment is very high, and therefore, is not used in routine work.

Fluorescent probes have been used to evaluate different steps of the freezing process (Blanc *et al.* 1991), and compare modifications in freezing (Kneissl 1993) or thawing techniques (Borg *et al.* 1997). The dual SYBR-14/PI stain has been used to assess quality of frozen-thawed stallion semen. Live spermatozoa emit green fluorescence (SYBR-14 +), and dead ones emit red colour (PI+). There was a negative correlation ($r = -0.49$) between the percentage of rapidly moving spermatozoa as estimated by HTM and the percentage of spermatozoa emitting red fluorescence (PI+). In contrast, a positive correlation ($r = 0.35$) was found between the percentage of rapid sperm and those emitting green fluorescence (Magistrini *et al.* 1997). Highly significant correlations were seen between MOT (Strömberg-Mika-Cell-Motion-Analysis-System) and intact spermatozoa, when frozen-thawed stallion semen was stained with (CFDA/PI) (Kneissl 1993). Motility of frozen-thawed stallion semen (VCL, MOT and ALH) was significantly correlated with degree

of degradation of the plasma membrane as evaluated by FITC-Con-A. Addition of glycerol caused significant reductions in VCL and ALH and increased the proportion of damaged spermatozoa, but the most pronounced changes in motility were observed after freezing and thawing (Blanc *et al.* 1991). In another study, FITC-PSA with ethidium homodimer as a counterstain was used to evaluate acrosomal status of stallion semen. Freezing and thawing resulted in a high percentage of acrosome-reacted or -damaged sperm and a significant decrease in sperm viability, suggesting an enhanced level of sperm capacitation-like changes or membrane damage (Bedford *et al.* 2000). When stallion semen samples with known percentages of acrosome-damaged spermatozoa were incubated with PSA, a positive correlation (0.98) was found between the percentage of spermatozoa bound to PSA and the percentage of acrosome-damaged spermatozoa (Farlin *et al.* 1992).

Studies on integrity of plasma and acrosomal membranes of frozen-thawed sperm have increased in the past years. It remains to be seen how well membrane integrity correlates with fertility results. Flow cytometric evaluation of viability of frozen-thawed PI-stained stallion (5 stallions) spermatozoa correlated with the fertility ($r = 0.68$) of 40 mares (80 cycles), and was better ($p < 0.05$) than other methods (MOT, hamster oocyte penetration) (Wilhelm *et al.* 1996).

Monoclonal antibodies and indirect immunolabelling techniques

A primary antibody specific for an acrosomal antigen can be used to evaluate integrity of acrosomal membranes. The antigen is localized at the inner surface of the outer acrosomal membrane. Only cells with damaged plasma and acrosomal membranes will bind primary antibody and demonstrate fluorescence after exposure to a secondary antibody (anti-mouse

IgG-FITC) when viewed by epifluorescence microscopy. In a German study, Spermac and immunohistochemical staining with monoclonal antibody were compared in the evaluation of acrosomes of frozen-thawed stallion sperm. Significantly more damaged acrosomes were diagnosed by Spermac (31%) as compared with monoclonal antibody (25%) (Schrop 1992). Wöckener & Schubert (1993) concluded that immunohistochemical staining with monoclonal antibody was superior to conventional staining techniques (Spermac and Karas) in assessing acrosomal status of frozen stallion semen.

Hypo-osmotic swelling test (HOS)

When spermatozoa are suspended in a hypo-osmotic solution, water will enter the spermatozoon in an attempt to attain osmotic equilibrium. This increases the volume of the cell, thereby reducing the initial length of the flagellum, and the plasma membrane bulges (Drevius & Eriksson 1966). The influx of water only occurs in the tail region and creates different types of curls. The appearance of a curl in the tail of a sperm is a sign that water has been transported in a physiological manner into the cell to reach osmotic equilibrium. This indicates an intact flagellar membrane (Colenbrander *et al.* 1992).

Nie and Wenze (2001) recommended that 100 μ l of stallion semen is added to 1 ml of 100 mOsm sucrose solution and incubated at 37°C for 60 min. They found the test to be simple, accurate and consistent with good reliability and repeatability. De Albuquerque Lagares (1995) saw vesicles in stallion sperm tails most frequently, when the osmolality was between 150 and 100 mOsm and Neild *et al.* (1999) between 100 and 25 mOsm. When testing 156 ejaculates from 13 stallions, a significant positive correlation was obtained between HOS and fertilization rate (Albuquerque Lagares 1995).

Resistance of stallion spermatozoa to hyperosmotic stress (600 to 4000 mOsm) was not useful in the evaluation of frozen-thawed stallion semen (Caiza de la Cueva *et al.* 1997). Several semen evaluation methods were applied in the assessment of fresh and frozen stallion semen in a French study. HOS was performed on fresh semen immediately after collection, and after an incubation of 4 h and 6 h at 37°C in the presence or absence of seminal plasma. After freezing and thawing, the HOS-test was carried out at 0 h and after an incubation of 4 h at 37°C, and after a storage of 7 days at 4°C. The HOS-test applied immediately after semen collection was highly correlated with MOT and ATP levels after thawing ($r > 50$) at 0 and 4 h and with MOT after 7 days. The authors suggested that HOS applied after collection of fresh semen is the best predictive test of the freezability of stallion semen (Vidament *et al.* 1998). Katila *et al.* (2000b) tested commercially used frozen semen from 31 stallions and compared results with foaling rates of 1085 mares. The HOS-test was carried out using a 100 mOsm solution and an incubation of 45 min at 37°C. A significant correlation was found between foaling rate and HOS-test performed on sperm immediately after thawing or after an incubation of 3 h at 37°C.

Filtration tests

When stallion sperm (fresh, freeze-damaged, uterine-inoculated) were filtered through cotton, glass wool (GW) and Sephadex (S) filters, the results indicated that spermatozoa with acrosome-damaged or -reacted sperm were trapped by GW filters. Spermatozoa with capacitation-like changes (uterine-inoculated sperm) were trapped by S-filters (Samper & Crabo 1993). In filtration of frozen-thawed semen of 9 stallions, significant correlations were obtained between the pregnancy rate per cycle (177 mares) and the percentage of sperm pass-

ing through the filters (GWS, $r = 0.93$ and S, $r = 0.84$) (Samper *et al.* 1991). If Sephadex traps capacitated spermatozoa, this finding would indicate that capacitation of spermatozoa is a problem with frozen-thawed sperm. GW-filtered human spermatozoa showed an increased capability to penetrate zona-free hamster oocytes (Rana *et al.* 1989). Motility did not account for the improved penetrability. When the filtered spermatozoa were diluted with nonviable spermatozoa, the improved oocyte penetration disappeared. Thus, it was concluded that the removal of nonviable spermatozoa may, at least, in part, be responsible for this effect (Rana *et al.* 1989). The results of Samper *et al.* (1991) and Samper & Crabo (1993) look promising, but, so far, filtration tests have not gained widespread acceptance. Vidament *et al.* (1998) considered GWS-filtration to be unreliable in the evaluation of frozen-thawed stallion semen, because 75% of the variance was due to error (straws, tubes, ejaculates). However, this statement was not substantiated with fertility results.

Biochemical tests

Cells with membrane damage lose essential metabolites and enzymes. Numerous enzymes have been determined in semen of several species, most often bulls and boars. These include aspartate-aminotransferase (AT-ase), fumarase, isocitrate dehydrogenase, aconitase, arylsulphatases (AS), Na^+/K^+ -ATPase, glutamic oxaloacetic transaminase (GOT), lactic dehydrogenase (LDH), cholinesterase, acid phosphatase and alkaline phosphatase (Brown *et al.* 1971, Risse 1990). AS-ases are present in the acrosome of the intact sperm cell and in seminal plasma. Membrane damage to the mid-piece results in release of AT-ase to the seminal plasma. As a result, ATP production is blocked, immobilising the sperm cell (Colenbrander *et al.* 1992). Kosiniak (1988) has advocated the

use of AT-ase as a good predictor of stallion semen freezability, suggesting that the higher the enzyme levels, the lower the motility after thawing. However, this was neither statistically analysed nor substantiated by fertility trials.

Acrosin is a proteolytic enzyme present in the acrosome and thought to be important in acrosome reaction, sperm-zona binding and zona penetration. Ball *et al.* (1997) determined acrosin amidase activity from raw semen, from semen extended in freezing extender and from frozen-thawed stallion semen. Acrosin activity increased with sperm concentration ($r^2 = 0.75$, $p < 0.001$), and the stallion and the ejaculate within stallion had significant effect on acrosin activity ($p < 0.001$). The addition of freezing medium increased activity, but no significant changes after freezing occurred (Ball *et al.* 1997). Vieira (1980) identified acrosin activity in stallion semen before and after freezing by means of a gelatine substrate method. Acrosin activity was detected by the presence of halos around single sperm, resulting from localized proteolytic digestion of gelatin. Morphological alterations of the acrosome and acrosin activity were correlated ($r = 0.9$, $p < 0.05$) in stallions only after a sexual rest of 6 months (Vieira 1980).

GOT is an intracellular enzyme with limited usefulness due to its presence in high concentrations in cytoplasmic droplets (Vieira 1980). After freezing and thawing of boar semen, a heterospermic index was correlated with the following *in vitro* tests: spermatozoa with acrosin-activity (0.38), extracellular GOT (0.54), intracellular GOT (-0.57) and motility (0.50) at 7 h post-thaw (Hammit *et al.* 1989). The authors pointed out that the extracellular GOT present immediately following ejaculation should be determined along with the GOT following freezing and thawing. The prefreeze GOT-values are then subtracted from post-thaw GOT-values because boars differ greatly in extracellular GOT before freezing.

The intact sperm cell has a relatively high content of ATP. If membranes are defective, the nucleotide phosphates will leak out of the cell into the seminal plasma and be hydrolyzed. ATP/ADP/AMP measurements in stallion sperm provide information on membrane viability (Colenbrander *et al.* 1992). Intracellular ATP content reflects mitochondrial activity of the stallion spermatozoon and can be determined by bioluminescence (Vidament *et al.* 1998). In their study, ATP and HOS were correlated shortly after semen collection, after 6 h survival at 37°C and after 4 h survival post-thaw at 37°C. The integrity of the plasma membrane of the flagellum seems to be essential for maintaining the mitochondrial activity and the ATP content (Vidament *et al.* 1998). In fresh and frozen stallion semen, ATP content was correlated with objective motility ($r = 0.92$) and velocity ($r = 0.87$) ($p < 0.05$) (Rodriguez & Bustos-Obregón 1996). The ATP content of the frozen-thawed stallion sperm was reduced 50% from the concentration in fresh semen (Rodriguez & Bustos-Obregón 1996).

Determinations of enzyme concentrations in semen have been practised for a long time. They are simple, rapid and inexpensive to do. On the other hand, they are prone to errors. It is necessary to select an enzyme found only in sperm cells. In addition to spermatozoa, enzymes can be present in cytoplasmic droplets, seminal plasma, and organic extenders. No convincing results have yet been presented that would favour the use of enzyme determinations in assessing pre- and post-thaw semen quality.

Sperm oocyte interactions

In all species, penetration of the oocyte by sperm requires motility, intact receptor proteins on the sperm to bind to the zona pellucida, and the ability to undergo an acrosome reaction and bind to the plasma membrane of the oocyte. Different *in vitro* penetration assays have been

developed to address each of these attributes (Graham 1997).

Zona pellucida (ZP) sperm binding

Zona penetration assays evaluate sperm motility, zona binding and penetration, sperm capacitation, and the acrosome reaction (Graham 1997). Capacitated spermatozoa from 3 fertile and 3 subfertile stallions were incubated with frozen-thawed equine oocytes (Meyers *et al.* 1996). The total number of ZP-bound spermatozoa was higher for fertile than for subfertile stallions ($p < 0.05$). Similarly, the percentage of acrosome reactions in ZP-bound spermatozoa was higher for the 3 fertile stallions than for the 3 subfertile stallions ($p < 0.05$) (Meyers *et al.* 1996).

Salt-stored equine oocytes maintain spermatozoal receptors on the ZP and can be used in sperm binding assays (Malchow & Arns 1995). When salt-stored equine oocytes were used, binding of spermatozoa from some subfertile stallions appeared to be lower than for fertile stallions, but variation was present. One of the reasons for such discrepancies might be differences in the oocytes and in their ZP (Pantke *et al.* 1995). In fact, immature oocytes bind fewer spermatozoa than oocytes in metaphase stage. The final stage of oocyte maturation is accompanied by some changes in the ZP (Mlodawska *et al.* 2000).

Hemizona assay (HZA)

In the HZA, the 2 matched zona hemispheres created by bisection are functionally equal surfaces, allowing for a controlled comparison of sperm binding. Thus, the variation in binding capacity between individual ZP is eliminated. The binding capacity of two semen samples to matching hemizonae can be compared. When semen samples from 22 stallions with known fertility data were tested on salt-stored hemizonae, there was a significant relationship

($p < 0.0001$) between the mean number of spermatozoa bound to matching hemizonae and the fertility indices of stallions from each stud farm (Fazeli *et al.* 1995).

In another Dutch study, ejaculates from 5 stallions were split into two samples: one was frozen and the other stored and chilled for 24 h. Equine oocytes were bisected, and one hemizona incubated with the chilled semen and the matching half incubated with frozen semen. Four oocytes were used per stallion. There was a significant difference in sperm binding between chilled and frozen-thawed samples (50 ± 4 and 41 ± 4 , respectively). The extent of the difference varied markedly between stallions (Parlevliet *et al.* 1994).

Zona-free hamster oocyte penetration test (HOPT)

In vitro penetration of zona-free hamster oocytes provides information about the capability of sperm that have already undergone the acrosome reaction to penetrate the oocyte. Multiple spermatozoa can penetrate the heterologous hamster oocyte (Graham 1997). In a Polish study, fresh hamster oocytes were incubated for 3 to 4 h with acrosome-reacted stallion spermatozoa. No conclusive relationship was established between sperm motility and the percentage of penetrated zona-free hamster oocytes (Okolski *et al.* 1987). In another study, frozen-thawed zona-free hamster oocytes (20/ejaculate) and acrosome-reacted stallion spermatozoa (fresh and cooled for 24 or 72 hours) were incubated for 8 min. The ability of sperm to penetrate zona-free hamster oocytes was shown to decrease with increased storage time of semen (Padilla *et al.* 1991). Penetration of zona-free hamster oocytes by frozen boar sperm was markedly reduced compared with fresh and liquid-stored semen (Clarke & Johnson 1987). Neither the percentage of penetrated zona-free hamster oocytes nor the average number of

spermatozoa penetrating each hamster oocyte were correlated with fertility, when cryopreserved stallion spermatozoa were tested (Wilhelm *et al.* 1996).

In vitro fertilization (IVF)

IVF has proven to be a reliable test for sperm quality and fertilizing capacity in human fertility clinics, but being an invasive and expensive procedure, it cannot be routinely used as a semen evaluation test (Yavetz *et al.* 1995). IVF has been successfully used to differentiate between frozen semen from low- and high-fertility bulls (Larsson *et al.* 1994). Unfortunately, IVF-techniques are not sufficiently advanced in horses to be used for this purpose.

The disadvantages of sperm penetration techniques are the time and expense needed, and that very few sperm are actually evaluated. The in vitro conditions are likely to be quite different from the in vivo environment (Graham 1997).

Progesterone-induced acrosome reaction

Mammalian sperm that have completed capacitation are capable of undergoing the acrosome reaction in response to a number of stimuli, e.g. progesterone. Progesterone in mare follicular fluid induces the acrosome reaction in capacitated stallion spermatozoa. The reaction is mediated by a plasma membrane progesterone receptor (Cheng 1997). The percentage of spermatozoa with exposed progesterone receptors was highly correlated to fertility of stallions (Rathi *et al.* 2000). Sperm from stallions classified as fertile on the basis of breeding history had higher percentages of progesterone-induced acrosome reactions in comparison with stallions classified as subfertile (Meyers *et al.* 1995). The test has not been applied to frozen semen.

Other tests

In humans, the cervical mucus penetration test (Morrow *et al.* 1992) and the microelectrophoretic motility test (Glander & Herold 1991) have been applied to study quality of fresh and frozen semen, but neither of these test have been used in horses.

Sperm chromatin structure assay (SCSA)

Chromatin in a spermatozoon is condensed to one-sixth of the volume in somatic cells. Normal sperm development leads to a chromatin structure in which the DNA in situ is resistant to denaturation, whereas the DNA of spermatozoa with an abnormal chromatin structure is susceptible to denaturation in situ. The percentage of cells with abnormal chromatin and the extent of the abnormality can be detected by the SCSA carried out in flow cytometry (Evenson *et al.* 1994). Subfertile stallions had a higher percentage of cells outside the main population (COM-P α _v) than normal stallions (Kenney *et al.* 1995). In swine, SCSA correctly predicted both high- and low-fertility boars based on a ratio of offspring as deviated from the theoretical percentage (Evenson *et al.* 1994). However, the boar sperm chromatin structure was unaltered by direct freezing on dry ice or in liquid nitrogen with or without different types of extenders. The loss of sperm fertility potential after freezing/thawing is due to factors other than damage to sperm chromatin structure, which is very resistant (Evenson *et al.* 1994). Thus, SCSA is not suitable for evaluation of effects of freezing on sperm, but can be used to evaluate the COMP α of the DNA of frozen semen.

Resazurin reduction test

A resazurin reduction test has been useful in determining the fertility potential of bovine spermatozoa. Metabolically active sperm reduce resazurin (blue) to pink and upon further reduction to white. Assessment of the reduction

from blue to pink allowed for the identification of 88% of the potentially low and 94% of the high-fertility samples (Dart *et al.* 1994). The resazurin reduction test performed immediately after thawing of frozen stallion semen had no correlation with fertility (Katila *et al.*, 2000a).

Transmigration rate

The transmigration rate (TMR%) has been used to compare motility of fresh and frozen-thawed stallion sperm. The rate is defined as the percentage of spermatozoa which within 3 min will migrate from the sample chamber through a membrane having pores of 8 μ m against a flow (5 ml/h) of buffer medium to the goal chamber. There was a highly significant correlation between TMR, motility and the percentage of viable cells (Renner *et al.* 1992). However, since TMR was not reported for fresh semen, it is not possible to know how well the test would reflect changes which have taken place during the freezing and thawing processes. The test has not been correlated with fertility.

Conclusions

Although motility evaluation has its limitations, it should be performed to obtain a minimum threshold value. In many laboratories, a minimum progressive motility of 30% is required. Subjective evaluation by a trained person using a good phase contrast microscope is adequate for routine evaluations. In scientific experiments, subjective evaluation has been replaced by the use of objective computerized image analysers. It is clear that freezing and thawing processes cause premature capacitation and acrosome reaction of spermatozoa, damage membranes and kill cells. Not all of these changes are reflected in motility, but sperm motility is a readily assayed barometer of relative cell health. Membrane and particularly acrosome integrity should be evaluated by fluo-

rescent probes. Other tests are not in routine use, although some of them might have potential in the evaluation of frozen semen. HOS-test, particularly, could be a useful test, and it is simple to perform. At the moment, it is obvious that we need to combine several tests for fertility evaluation of frozen-thawed stallion semen. Much research is still needed in this field if we are to be able to increase the use of frozen stallion semen and get good pregnancy rates. Reliable in vitro semen evaluation methods are a prerequisite to the development of freezing and thawing techniques. Quality control of frozen semen by dependable laboratory methods is necessary before semen is distributed to the field.

Acknowledgements

The author wishes to thank professor R.M. Kenney, ass. professor Stewart Meyers, and Paul Loomis, president of Select Breeders Service, Inc., for reading the manuscript critically and for providing excellent comments.

References

- Abraham-Peskir JV, Chantler E, Uggerhoj E*: Significance of plasmalemma disruption in bovine and equine spermatozoa. *Theriogenology* 2000, *54*, 1075-1086.
- Agarwal A, Ozturk E, Loughlin KR*: Comparison of semen analysis between the two Hamilton-Thorn semen analysers. *Andrologia* 1992, *24*, 327-329.
- Amann RP*: Can the fertility potential of a seminal sample be predicted accurately? *J. Andrology* 1989, *10*, 89-98.
- Ball BA, Fagnan MS, Dobrinski I*: Determination of acrosin amidase activity in equine spermatozoa. *Theriogenology* 1997, *48*, 1191-1198.
- Bataille B, Magistrini M, Palmer E*: Analyse objective de la mobilite du sperme congele-decongele d'etalon. Essai de correlation avec la fertilité. (Objective determination of sperm motility in frozen-thawed stallion semen. Correlation with fertility). *Anim. Breed. Abstr.* 1990, 96-106.
- Bedford SJ, Varner DD, Meyers SA*: Effects of cryopreservation on the acrosomal status of stallion spermatozoa. *J. Reprod. Fert.* 2000, Suppl. 56, 133-140.
- Bielanski W*: The evaluation of stallion semen in aspects of fertility control and its use for artificial insemination. *J. Reprod. Fert.* 1975, Suppl 23, 19-24.
- Bielanski W, Kaczmarek F*: Morphology of spermatozoa in semen from stallions of normal fertility. *J. Reprod. Fert.* 1979, Suppl 27, 39-45.
- Blach EL, Amann RP, Bowen RA, Frantz D*: Changes in quality of stallion spermatozoa during cryopreservation: plasma membrane integrity and motion characteristics. *Theriogenology* 1989, *31*, 283-298.
- Blanc G, Magistrini M, Palmer E*: Use of Concanavalin A for coating the membranes of stallion spermatozoa. *J. Reprod. Fert.* 1991, Suppl 44, 191-198.
- Borg K, Colenbrander B, Fazeli A, Parlevliet J, Malmgren L*: Influence of thawing method on motility, plasma membrane integrity and morphology of frozen-thawed stallion spermatozoa. *Theriogenology* 1997, *48*, 531-536.
- Boyle MS*: Artificial insemination: assessing stallion semen quality after freezing. *Eq. Vet. J.* 1996, *28*, 5-6.
- Brown KI, Crabo BG, Graham EF, Pace MM*: Some factors affecting loss of intracellular enzymes from spermatozoa. *Cryobiology* 1971, *8*, 220-224.
- Budworth PR, Amann RP, Chapman PL*: Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. *J. Andr.* 1988, *9*, 41-54.
- Burns PJ, Reasner DS*: Computerized analysis of sperm motion: effects of glycerol concentration on the cryopreservation of equine spermatozoa. *J. Equine Vet. Sci.* 1995, *15*, 377-380.
- Caiza de la Cueva FI, Pujol MR, Rigau T, Bonet S, Miró J, Briz M, Rodriguez-Gill JE*: Resistance to osmotic stress of horse spermatozoa: The role of ionic pumps and their relationship to cryopreservation success. *Theriogenology* 1997, *48*, 947-968.
- Casey PJ, Hillman RB, Robertson KR, Yudin AI, Liu IKM, Drobnis EZ*: Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. *J. Andrology* 1993, *14*, 289-297.
- Cheng F-P*: The acrosome reaction in stallion spermatozoa. Thesis, Utrecht 1997.

- Cheng F-P, Fazeli A, Voorhout WF, Marks A, Bevers MM, Colenbrander B: Use of PNA (Peanut Agglutinin) to assess the acrosomal status and the zona pellucida induced acrosome reaction in stallion spermatozoa. *J. Andr.* 1996, 17, 674-682.
- Christensen P, Parlevliet JM, van Buiten A, Hyttel P, Colenbrander B: Ultrastructure of fresh and frozen-thawed stallion spermatozoa. *Biol. Reprod.* 1995, Mono 1, 769-777.
- Clarke RN, Johnson LA: Effect of liquid storage and cryopreservation of boar spermatozoa on acrosomal integrity and the penetration of zona-free hamster ova in vitro. *Gamete Res.* 1987, 16, 193-204.
- Colenbrander B, Fazeli AR, van Buiten A, Parlevliet J, Gadella BM: Assessment of sperm cell membrane integrity in the horse. *Acta vet. Scand.* 1992, Suppl 88, 49-58.
- Comhaire FH, Huysse S, Hinting A, Vermeulen L, Schoonjans F: Objective semen analysis: has the target been reached? *Human Reprod.* 1992, 7, 237-241.
- Dart MG, Mesta J, Crenshaw C, Ericsson SA: Modified resazurin reduction test for determining the fertility potential of bovine spermatozoa. *Arch. Andr.* 1994, 33, 71-75.
- Davis RO, Gravance CG, Casey PJ: Automated morphometric analysis of stallion spermatozoa. *Am. J. Vet. Res.* 1993, 54, 1808-1811.
- De Albuquerque Lagares M: Bestimmung der osmotischen Resistenz von Hengstsamenzellen (The determination of the osmotic resistance of stallion spermatozoa). Thesis, Hannover 1995.
- Dowsett KF, Pattie WA: Characteristics and fertility of stallion semen. *J. Reprod. Fert.* 1982, Suppl 32, 1-8.
- Drevius LO, Eriksson H: Osmotic swelling of mammalian spermatozoa. *Exp. Cell Res.* 1966, 42, 136-156.
- Eriksson B, Juonala T, Andersson M, Rodriguez-Martines H: Viability of frozen-thawed boar semen. *Proc. 50th Anniv. ICAR* 1998, 526.
- Evenson DP, Thompson L, Jost L: Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. *Theriogenology* 1994, 41, 637-651.
- Farlin ME, Jasko DJ, Graham J, Squires EL: Assessment of *Pisum sativum* agglutinin in identifying acrosomal damage in stallion spermatozoa. *Molec. Reprod. Dev.* 1992, 32, 23-27.
- Farrell PB, Foote RH, Zinaman MJ: Motility and other characteristics of human sperm can be measured by computer-assisted sperm analysis of samples stained with Hoechst 33342. *Fert. & Ster.* 1996, 66, 446-453.
- Fazeli AR, Steenweg W, Bevers M.M., Broek Jv, Bracher V, Parlevliet J, Colenbrander B: Relation between stallion sperm binding to homologous hemizona and fertility. *Theriogenology* 1995, 44, 751-760.
- Glander HJ, Herold W: Influence of cryopreservation on the microelectrophoretic motility (EPM) of human spermatozoa. *Andrologia* 1991, 23, 263-267.
- Graham JK: Response of sperm to freezing and analysis of sperm. *Proc. Ann. Meet. Soc. Theriog.* 1997, 170-177.
- Gravance CG, Garner DL, Baumber J, Ball BA: Assessment of equine sperm mitochondrial function using JC-1. *Theriogenology* 2000, 53, 1691-1703.
- Hammit DG, Martin PA: Correlations among assays of porcine semen quality following cryopreservation. *Theriogenology* 1989, 32, 369-384.
- Hermenet MJ, Sawyer HR, Pickett BW, Amann RP, Squires EL, Long PL: Effect of stain, technician, number of spermatozoa evaluated and slide preparation on assessment of spermatozoal viability by light microscopy. *J. Equine Vet. Sci.* 1993, 13, 449-455.
- Jasko DJ: Evaluation of stallion semen. In: Blanchard TL, Varner DD (eds), *Vet. Clin. NA, Equine Practice* 1992, 8, 129-148.
- Jasko DJ, Lein DH, Foote RH: Determination of the relationship between sperm morphologic classifications and fertility in stallions: 66 cases (1987-1988). *J. Am. Vet. Med. Assoc.* 1990a, 197, 389-394.
- Jasko DJ, Lein DH, Foote RH: A comparison of two computer-automated semen analysis instruments for the evaluation of sperm motion characteristics in the stallion. *J. Andr.* 1990b, 11, 453-459.
- Jasko DJ, Little TV, Lein DH, Foote RH: Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). *J. Am. Vet. Med. Assoc.* 1992, 200, 979-985.
- Katila T, Kuisma P, Andersson M: Evaluation of frozen stallion semen. In: Allen WR, Wade JF (eds): *Proceeding of the First Meeting of the European Equine Gamete Group (EEGG)*. Havemeyer Foundation Monograph Series No 1, 2000, 19-21a.
- Katila T, Koskinen E, Andersson M: Evaluation of

- frozen-thawed semen. A Dorothy Russell Have-meyer Foundation Workshop Advanced Current Topics in Stallion Veterinary Practice, 2000, 54-56b.
- Kenney RM, Evenson DP, Garcia MC, Love CC:* Relationships between sperm chromatin structure, motility, and morphology of ejaculated sperm, and seasonal pregnancy rate. *Biol. Reprod.* 1995, Mono 1, 647-653.
- Kenney RM, Hurtgen J, Pierson R, Witherspoon D, Simons J:* Theriogenology and the equine, part II: the stallion. *J. Soc. Theriog.* 1893, 9.
- Kneissl S:* Tiefgefrierkonservierung von Pferdesperma: Einfluss der Samenentnahmetechnik, Zentrifugation, Konfektionierungsform und Einfriermethode auf die Motilität und Membranintegrität der Samenzellen. (Cryopreservation of stallion semen: The influence of semen collection techniques, centrifugation, packaging forms, and methods of freezing on the motility and plasma membrane integrity of spermatozoa) Thesis Hannover, 1993.
- Kosiniak K:* The evaluation of stallion semen in aspects of freezability control and its use for practice. *Egypt. J. Vet. Sci.* 1988, 25, 1-19.
- Larsson B, Andersson M, Aalto J, Shamsuddin M, Rodriguez-Martinez H:* A comparison between different methods for estimation of the fertility in AI young bulls. XVII Nordic Vet. Congr. Reykjavik, Iceland 1994, 216-217.
- Loomis P:* Artificial insemination of horses: Where is it going? *Soc. Theriogenology, Proc. Ann. Conf.* Nashville, Tennessee, Sept. 22-24, 1999, 325-336.
- Magistrini M, Guitton E, Levern Y, Nicolle JC, Vidament M, Kerboeuf D, Palmer E:* New staining methods for sperm evaluation estimated by microscopy and flow cytometry. *Theriogenology* 1997, 48, 1229-1235.
- Malchow NM, Arns MJ:* Influence of salt storage on equine zonae pellucidae: electrophoretic properties and interaction with spermatozoa. *Biol. Reprod.* 1995, Mono 1, 671-679.
- Malmgren L:* Assessing the quality of raw semen: a review. *Theriogenology* 1997, 48, 523-530.
- Mennick PE:* Equine semen cryopreservation: An update. *Soc. Theriogenology, Proc. Ann. Meeting*, September 17-20, 1997, Montreal, Quebec, Canada: 161-169.
- Meyers SA, Liu IKM, Overstreet JW, Drobnis EZ:* Sperm-zona pellucida binding and zona-induced acrosome reactions in the horse: Comparisons between fertile and subfertile stallions. *Theriogenology* 1996, 46, 1277-1288.
- Meyers SA, Overstreet JW, Liu IKM, Drobnis EZ:* Capacitation in vitro of stallion spermatozoa: Comparison of progesterone-induced acrosome reactions in fertile and subfertile mares. *J. Andr.* 1995, 16, 47-54.
- Mlodawska W, Palmer E, Duchamp G, Okólski A, Bezar J:* Zona pellucida-sperm binding assay for equine oocytes. *J. Reprod. Fert.* 2000, Suppl. 56, 423-429.
- Morrell JM:* Applications of flow cytometry to artificial insemination: a review. *Vet. Rec.* 1991, 129, 375-378.
- Morrow A, Drudy L, Gordon A, Harrison RF:* Evaluation of bovine cervical mucus penetration as a test of human spermatozoal function for an in vitro fertilization programme. *Andrologia* 1992, 24, 323-326.
- Müller Z:* Fertility of frozen equine semen. *J. Reprod. Fert.* 1982, Suppl 32, 47-51.
- Müller Z:* Practicalities of insemination of mares with deep-frozen semen. *J. Reprod. Fert.* 1987, Suppl 35, 121-125.
- Neild D, Chaves G, Flores M, Mora N, Beconi M, Agüero A:* Hypoosmotic test in equine spermatozoa. *Theriogenology* 1999, 51, 721-727.
- Nie GJ, Wenzel JGW:* Adaptation of the hypoosmotic swelling test to assess functional integrity of stallion spermatozoal plasma membranes. *Theriogenology* 2001, 55, 1005-1018.
- Oettle EE:* Using a new acrosome stain to evaluate sperm morphology. *Vet. Med.* 1986, 81, 263-266.
- Okólski A, Babusik P, Tischner M, Lietz W:* Evaluation of mare oocyte collection methods and stallion sperm penetration of zona-free hamster ova. *J. Reprod. Fert.* 1987, Suppl 35, 191-196.
- Padilla AW, Tobbac C, Foote RH:* Penetration of frozen-thawed, zona-free hamster oocytes by fresh and slow-cooled stallion spermatozoa. *J. Reprod. Fert.* 1991, Suppl. 44, 207-212.
- Palmer E, Magistrini M:* Automated analysis of stallion semen post-thaw motility. *Acta vet. Scand.*, 1992, Suppl 88, 137-152.
- Pantke P, Hyland JH, Galloway DB, Liu DY, Baker HWG:* Development of a zona pellucida sperm binding assay for the assessment of stallion fertility. *Biol. Reprod.* 1995, Mono 1, 681-687.
- Papaioannou KZ, Murphy RP, Monks RS, Hynes N, Ryan MP, Boland MP, Roche JF:* Assessment of viability and mitochondrial function of equine spermatozoa using double staining and flow cy-

- ometry. *Theriogenology* 1997, 48, 299-312.
- Parlevliet JM, Fazeli AR, Steenweg W, Bevers MM, Colenbrander B*: The effect of storage methods on the sperm zona binding in the horse. 6th Int. Symp. Equine Reprod., Caxambu, Brazil, Programme and miniposters 1994, 171-172.
- Pickett BW, Squires EL, McKinnon AO*: Procedures for collection, evaluation and utilization of stallion semen for artificial insemination. Anim. Reprod. Lab., Colorado State University, Fort Collins, 1987.
- Rana N, Jeyendran RS, Holmgren WJ, Rotman C, Zaneveld LJD*: Glass wool-filtered spermatozoa and their oocyte penetrating capacity. J. in Vitro Fertil. and Embryo Transfer 1989, 6, 280-284.
- Rathi R, Nielen M, Cheng FP, van Buiten A, Colenbrander B*: Exposure of progesterone receptors on the plasma membranes of stallion spermatozoa as a parameter for prediction of fertility. J. Reprod. Fert. 2000, Suppl. 56, 87-91.
- Renner C, Gehring W, Holzmann A, Failing K*: Korrelationen spermabiologischer Merkmale in nativen und aufgetauten Hengstjakulaten unter Berücksichtigung der Transmigration. (Correlations of semen characters in fresh and thawed stallion ejaculates, with consideration of transmigration) *Reprod. Dom. Anim.* 1992, 27, 148-153.
- Risse S*: Die bisherigen Möglichkeiten zur Einschätzung der Qualität und Fertilität von Spermienzellen. (Existing possibilities for evaluating sperm quality and fertility). Monatshefte Vet. Med. 1990, 45, 348-352.
- Rodriguez H, Bustos-Obregón E*: Seasonality and freezability vs routine parameters in stallion semen. *Histol. Histopathol.* 1996, 11, 427-430.
- Samper JC, Crabo BG*: Assay of capacitated, freeze-damaged and extended stallion spermatozoa by filtration. *Theriogenology* 1993, 39, 1209-1220.
- Samper JC, Hellander JC, Crabo BG*: Relationship between the fertility of fresh and frozen stallion semen and semen quality. *J. Reprod. Fert.* 1991, Suppl 44, 107-114.
- Schrop H*: Einfluss der Membranprotektiva Lecithin und Phenylmethansulfonylfluorid auf Motilität und Akrosomenintegrität im aufgetauten Samen; Beurteilung mittels indirekter Immunfluoreszenz im Vergleich zur Spermac-Färbung. (The membrane protective influence of lecithine and phenylmethansulfonyl fluoride on motility and acrosomal integrity of the frozen-thawed equine semen). Thesis, Hannover 1992.
- Squires EL, Amann RP, Pickett BW*: Preservation of stallion semen - Colorado experience. *Symp. Equine Reprod.*, Denmark 1987, 49-54.
- Tischner M*: Evaluation of deep-frozen semen in stallions. *J. Reprod. Fert.* 1979, Suppl 27, 53-59.
- Van Huffel XM, Varner DD, Hinrichs K, Garcia MC, Strzemienski PJ, Kenney RM*: Photomicrographic evaluation of stallion spermatozoal motility characteristics. *Am. J. Vet. Res.* 1985, 46, 1272-1275.
- Varner DD, Bowen JA, Johnson L*: Effect of heparin on capacitation/acrosome reaction of equine sperm. *Arch. Andr.* 1993, 31, 199-207.
- Varner DD, Schumacher J, Blanchard TL, Johnson L*: Diseases and Management of Breeding Stallions. American Veterinary Publications, Goleta, CA 1991b.
- Varner DD, Vaughan SD, Johnson L*: Use of a computerized system for evaluation of equine spermatozoal motility. *Am. J. Vet. Res.* 1991, 52, 224-230a.
- Vidament M, Cognard E, Yvon J-M, Sattler M, Palmer E, Magistrini M*: Evaluation of stallion semen before and after freezing. *Reprod. Dom. Anim.* 1998, 33, 271-277.
- Vidament M, Dupere AM, Julienne P, Evain A, Noue P, Palmer E*: Equine frozen semen freezability and fertility field results. *Theriogenology* 1997, 48, 907-917.
- Vieira RC*: Akrosinbestimmung an Pferfespermien unter Berücksichtigung bestimmter andrologischer Fragestellungen. (Acrosin determination on equine spermatozoa). Thesis, Hannover 1980.
- Voss JL, Pickett BW, Squires EL*: Stallion spermatozoal morphology and motility and their relationship to fertility. *J. Am. Vet. Med. Assoc.* 1981, 178, 287-289.
- Wilhelm KM, Graham JK, Squires EL*: Comparison of the fertility of cryopreserved stallion spermatozoa with sperm motion analyses, flow cytometric evaluation, and zona-free hamster oocyte penetration. *Theriogenology* 1996, 46, 559-578.
- Wöckener A, Schuberth HJ*: Freezing of maiden stallion semen-motility and morphological findings in sperm cells assessed by various staining methods including a monoclonal antibody with reactivity against an antigen in the acrosomal ground substance. *Reprod. Dom. Anim.* 1993, 28, 265-272.
- Yavetz H, Hauser R, Yogev L, Botchan A, Lessing JB, Homonnai ZT, Paz G*: Advanced methods for evaluation of sperm quality. *Andrologia* 1995, 27, 31-35.
- Ziegler R*: Computervideomikroskopische Beurteil-

ung von Hengstesperma vor und nach der Tiefgefrierkonservierung mit eidotterhaltigem Verdüner. (Computerised videomicrographic evaluation of stallion semen before and after freezing in a diluent containing egg yolk). Thesis, Hannover 1991.

Sammandrag

Bedömning av frys-tinad hingst sperma med hjälp av in vitro metoder. En översikt.

Denna översiktsartikel refererar olika in vitro undersökningsmetoder som använts på frys-tinad hingst sperma. Metodernas begränsningar, det praktiska utförandet och korrelationen med fertilitetsresultaten diskuteras. Få studier har gjorts för att undersöka korrelationen mellan fertiliteten hos fryst hingst sperma och laboratorietest. Det är svårt och dyrt att inseminera tillräckligt många ston för att erhålla sta-

tistiskt signifikanta resultat. Signifikanta, men låga korrelationer har kunnat påvisas mellan fölningsprocent och spermernas subjektiva motilitet efter en inkubationstid på 2 och 4 timmars vid 37°C samt med en hypo-osmotisk svällningstest efter 0 och 3 timmars inkubation. Signifikanta korrelationer har påvisats mellan dräktighetsprocent och viabilitet hos propidium-iodid färgad sperma som analyserats med en flowcytometer. Detsamma gäller för filtrationstester med glas-ull och Sephadex filter. Ingen korrelation mellan fertiliteten och motiliteten genast efter upptining har påvisats. Trots detta är den subjektiva motilitetsbedömningen med hjälp av ett ljusmikroskop den vanligaste undersökningsmetoden vid bedömningen av fryst hingstesperma. Datorassisterade automatiska spermieanalyser har ersatt ljusmikroskopi i forskningsprojekt, men än så länge har ingen kunnat demonstrera en korrelation mellan fryst hingstesperma och någon av de motilitetsparametrar som dessa instrument kan ge.

Reprints may be requested from: T. Katila, University of Helsinki, Saari Unit, Pohjoinen Pikatie 800, FIN-04920 Saarentaus. E-mail: tkatila@saari.vetmed.helsinki.fi, tel: +358-19-6851181, fax: +358-19-5295303.