Laboratory tests for sperm quality and fertility in stallions

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Swedish title: Laboratorietester som mått på spermiekvalitet och fertilitet hos hingst
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Animal Science – Bachelor degree project 15hp
Literature study
SLU, Uppsala 2009

Sammanfattning

Syftet med denna litteraturgenomgång var att presentera några av de vanligast förekommande laboratorietesterna som används idag vid utvärdering av spermakvalité hos hingstar. En granskning har också gjorts för att försöka utröna om det går att förutse hingstars fertilitet utifrån resultaten av dessa tester. De metoder som presenteras analyserar motilitet, morfologi och funktion hos spermierna i ett ejakulat. För att kunna fullfölja en befruktning krävs det att spermien uppfyller vissa kriterier. Detta för att kunna ta sig fram till äggcellen och smälta samman med denna och bilda ett embryo. En del av testerna görs även för att räkna ut hur stor dos av ejakulatet som behövs vid artificiell inseminering. Slutsatsen som kunde dras i denna litteraturgenomgång var att de laboratorietester som används idag endast ger en skattning av fertiliteten och ingen korrekt förutsägelse. Testerna verkar lämpa sig bättre för att upptäcka bristande fruktsamhet än för att utvärdera graden av fertilitet. Mer forskning inom detta område är önskvärt i framtiden, både vad gäller utveckling av laboratorietester med mer tillförlitliga resultat och ökad kännedom om spermiefunktion och befruktning.

Abstract

The purpose of this literature review was to describe some of the most commonly used laboratory tests for sperm quality in stallions and try to find out if the tests are reliable enough to predict fertility. The presented tests are used to evaluate motility, morphology and function of sperm cells in semen. To enable fertilization some traits has to be fulfilled. The sperm cell has to be able to reach the egg cell and fuse with it to achive embryo development. Some of the tests are also required to calculate the amount of semen needed for artificial insemination. In conclusion, this review shows that the laboratory tests used today only gives estimation, and no prediction, of fertility in stallions. The tests seem to detect fertility defects better than the rate of fertility. Further research in this area is desired in the future. Development of new tests with more reliable results and increased knowledge about the function of spermatozoa and fertilization is wanted to allow better prediction of fertility.

Introduction

Artificial insemination (AI) is frequently used in Swedish Horse breeding today. There were 219 stallions used in warmblood breeding in Sweden during 2008. Of these total 219 stallions only 15 stallions were used for natural mating (ASVH, 2009). Both fresh semen, cooled transported semen and frozen semen is used. Fresh semen is collected from the stallion at the stud farm and used for insemination during the same day. Alternatively the semen is cooled and sent to a breeding station where the mare is inseminated the following day. Frozen semen is more difficult to administrate. It has to be transported cold, it requires higher semen quality, more precise timing for AI and the fertility of the mare has to be sufficient (Colenbrander *et al.*, 2003). All this factors make AI with frozen semen more expensive than other methods.

AI has many advantages towards natural mating. An individual can be used for an additional number of females, due to dilution of ejaculate and the lower amount of semen needed for fertilization. The transportation of breeding animals is minimized since the semen can be transported instead. This also makes export and import of semen possible without geographic limitations. The risk for injuries and transmitting of diseases is also lowered (Lärn-Nilsson *et al.*, 2005). According to the Swedish Warmblood Association´s (ASVH´s) homepage in total 95,3% of all mares fertilized with warmblood stallions were inseminated with fresh, cool or frozen semen in 2008 (ASVH, 2009).

Other domestic animal species are often bred for optimized productivity where reproduction is a very important trait. The selection of males for good fertility is thus one of the main concerns. Horses, on the other hand, are primarily selected for breeding on other reasons such as pedigrees, performances or other desirable characteristics. Fertility of the stallion is therefore often of secondary consideration (Colenbrander *et al.*, 2003). This has led to a big variation in fertility among stallions. The breeding values for Swedish warmblood stallions do not include fertility. If the number of foals produced, as a percentage of the mares mated, is lower than 40% an evaluation of the stallion is made. The male can then be rejected as a breeding animal if the problem remains, e.g. fails to get mares pregnant in an acceptable rate (ASVH, 2009). Stallions with relatively low fertility can be tolerated in breeding due to other breeding goals than for production animals, and our ability to influence the fertility by high quality breeding management (Colenbrander *et al.*, 2003).

The most accurate way to evaluate stallion fertility is by mating a large number of normal mares and measuring the number of foals. Unfortunately this is an expensive and time consuming method (Rodriguez-Martinez, 2003). The ideal is therefore to predict fertility in advance, before the male is used in breeding. This is usually done by a physical examination of the stallion and a routine analysis of the semen quality. Unfortunately this procedure mostly identifies stallions with obvious defects that will influence their capacity to fertilize. It does not predict the level of fertility or identify sub-fertile animals (Colenbrander *et al.*, 2003). AI requires high quality semen, whether fresh, cooled, or frozen thus laboratory tests are needed for further evaluation of sperm function. It is also important to have knowledge about the correlation between semen quality and the likelihood of fertility to prevent time, resources and efforts to be wasted by using poor samples (Holt *et al.*, 2007).

The aim of this literature review was to describe some of the most commonly used laboratory tests for sperm quality in stallions and try to find out if the tests are reliable enough to predict fertility.

Stallion semen

The ejaculate of a stallion has a volume of 20-70 ml (Rodriguez-Martinez, 1996) and is composed of seminal plasma and sperm cells, also called spermatozoa. The seminal plasma is a secretion, rich in nutrients, produced in the accessory sex glands. The secretion affects the environment in the female sex organs and thereby enhances the viability and fertilization capacity of the sperm cells. A spermatozoon consists of head and tail, the upper part of the tail is called midpiece. The head contains the nucleus in which DNA is held and the acrosome, a specialized vesicle where enzymes are stored. These enzymes are synthesized during differentiation of spermatozoa and used during the penetration of the egg cell, also called the oocyte. The midpiece contains the sperm cell mitochondria which produce ATP. The tail is a single cilium and makes the spermatozoa mobile which is of importance for reaching the egg cell. A single plasma membrane encloses the whole spermatozoa (Sjaastad *et al.*, 2003).

The production of spermatozoa, the spermatogenesis, takes place in the testes. The spermatogenesis in domestic animals takes about 40-60 days from the first division of the stem cells to the release of mature spermatozoa. When the spermatozoa have been produced they are recived by the epididymis where they undergo their final maturation and develop motile capacity. The sperm cells are stored in epidedymis until transported to the female reproductive tract during mating. Spermatozoa and the seminal plasma are mixed when the sperm cells are transported from the epididymis (Sjaastad *et al.*, 2003).

Fertilization

Fertilization of the oocyte can not be done immediately after ejaculation. The membrane of the sperm cell has to be prepared before the fusion. This process is called capacitation and it occurs during the first 4-6 hours in the female reproductive tract. During fertilization the cell membranes of the spermatozoa and the oocyte fuses. This is done when the spermatozoa has penetrated the layers of cells protecting the oocyte. These layers are called corona radiata and zona pellucida. The enzymes in the acrosome are used during this process. When the penetration is completed the nucleus of the spermatozoa, containing the DNA, is transferred into the oocyte and a zygote will be created (Sjaastad *et al.*, 2003).

Why is evaluation of semen needed?

When semen has been collected from the stallion it will be evaluated. Volume, colour and potential contamination is analysed. Motility, morphology and the concentration of spermatozoa is also examined (Söderquist, 1996). The evaluation is needed to decide both semen quality and the number of AI doses which can be made out of it. Fertility is also estimated from the results (Foxcroft *et al.*, 2008). It is important to handle the semen with care and maintain a suitable temperature during the evaluation. Any change of temperature, cooling or heating, can affect the semen and lead to incorrect results (Estrada & Samper, 2007). If the spermatozoa has defects in motility or morphology it might be unable to reach the oocyte and thereby unable to fertilize. Some defects can be compensated by using a larger amount of semen during AI but unfortunately not all weaknesses can be overcome in this way. Traits that affect fertilization but do not affect motility or morphology are therefore more difficult to predict. This makes it of big importance to consider both compensatable traits and the traits that are not when estimating fertility (Foxcroft *et al.*, 2008).

Macroscopic evaluation

Macroscopic evaluation, performed with the naked eye, is applied to discover deviation in appearance of the semen. This is done shortly after the semen has been collected. The colour of the semen is observed, a normal stallion ejaculate should be white or grayish-white, other colours indicate defects (Estrada & Samper, 2007). The smell is also examined, stallion semen is normally odor neutral. If there is any odor it may indicate flaws in the sample (Sieme, 2009). The volume of the ejaculate is also measured. This amount is affected by a number of factors some of them are breed, age, season and collection frequency (Estrada & Samper, 2007).

Concentration of spermatozoa

The concentration of spermatozoa in semen can be counted in several ways. One method is by utilizing a hemocytometer, or counting chamber, and a microscope. It is a time consuming method but the result is considered more accurate and reliable then many other methods. Another common technique is using a spectrophotometer in which a beam of light is transmitted through the sample and measured by a phototube. The amount of light able to pass through the sample is correlated with the concentration of sperm cells in the sample (Evenson *et al.*, 1993). A disadvantage of this method is that all cells in the sample are evaluated, not only sperm cells, which can lead to an incorrect result. Knowledge about the concentration combined with the semen volume is a requirement for calculation of total sperm count of the ejaculate. From this number the amount of AI doses can be decided (Sieme, 2009).

Cytology examination

To assess if the semen is contaminated with bacteria a cytology examination can be done, a drop of raw semen is placed on a microscope slide and stained in several steps. After the staining the slide is rinsed with deionized water and dried before the evaluation. Neutrophils and other unwanted cells in the sample can now be seen and counted in a microscope. If the number of neutrophils reaches a certain quantity, it can be an indication of infection (Estrada & Samper, 2007).

Techniques for evaluation of sperm motility

There are many different techniques for evaluating motility of spermatozoa in semen. Which one to utilize depends on how experienced the user is, the wish for precision and repeatability and the equipment that are at hand. In most techniques the result is expressed as percentage motile sperm cells in the sample (Rouge, 2004). Evaluation of sperm cell motility is considered to be central for measuring the fertilizing capacity and provides an indication of how the sperm cells will performe in their natural environment (Varner, 2008).

Manual motility estimates

The evaluation of sperm motility can be made manually. A sample of diluted semen is examined with a microscope and the number of motile sperm is estimated (O´Connor *et al*., 1981). The dilution used consists of warm extender or buffered saline solution. This technique is easy to use and require minimal equipment. The major limitation is that the evaluator should be experienced to provide reliable estimates of motility (Rouge, 2004).

Track motility estimates

Preparation of samples for this technique is carried out in the same way as for manual estimation, as mentioned above. The sample is put in a microscope and the sperm cells are photographed, with an exposure time long enough for motile sperm cells to leave tracks across the image. Sperm cells with sufficient motility will leave straight tracks and cells with insufficient motility will leave abnormal or circular tracks. Non-motile sperm cells will be detected by leaving no tracks. If a digital camera is utilized during the process the images can be transferred to a computer and put on record. This technique is therefore objective and repeatable but it is more time consuming then manual estimates (Rouge, 2004). Evaluation procedures that are supported by computers are usualy called computer-assisted semen analyses (CASA). Special computer systems have been developed for this cause (Verstegen *et al.*, 2002). More about CASA will be discussed later in this review.

Techniques for evaluation of sperm morphology

During evaluation of sperm cell structure, morphology, the size and the shape of head, midpiece and tail are examined. Information about the sperm cell membranes and acrosome can also be gained. The result is expressed as percentage of sperm cells with normal morphology in the sample. There are always a number of morphologically abnormal sperm cells in an ejaculate. Fertility might be affected if the number is excessive (Rouge, 2004). Sperm cell abnormality can be classified in two different ways. One way is regarding to where in the sperm cell the defect is found. It is possible that more then one site is defective in the same sperm cell. The other way is to classify the flaws into primary, secondary and tertiary. Primary defects include failure during spermatogenesis, secondary include failure

during maturation of the sperm cell and tertiary include damages during or after ejaculation (Sieme, 2009).

Sperm morphology can be evaluated in many ways, one is by utilizing a microscope (Varner, 2008). In this method the sample is fixed with glutaraldehyde or formaldehyde which makes the durability longer (Rouge, 2004; Morrell *et al.*, 2008). Experienced technicians and a great deal of time are needed during this procedure. The sample can be examined in the microscope with or without staining (Sieme, 2009).

Staining techniques

Several different staining techniques have been developed for evaluation of sperm morphology (Varner, 2008). The staining is effective and uncomplicated to use. A droplet of semen is mixed with a drop of stain and observed in a microscope (Rouge, 2004). Some commonly used stains are eosin-nigrosin, eosin-aniline blue and different kinds of fluorescent staining (Sieme, 2009). Some stains examine morphology and assesses membrane integrity at the same time, a so called "live-dead" stain (Rouge, 2004).

Membrane integrity

Membrane integrity is a measurement of the amount of fluid transported into the spermatozoa across the plasma membrane. Evaluation of this trait is important since it is fundamental in the fertilization process (Estrada & Samper, 2007). Membrane integrity can be examined in a number of ways. One is using a classical staining technique were the membrane integrity of a normal, living sperm cell prevents the stain to emerge into the cell. It will therefore appear uncoloured (Varner, 2008). Dead sperm cells or those with membrane integrity losses will take up the stain and become coloured. The dead and defect cells can then be separated from normal and living ones (Rouge, 2004). One technique to detect the different cells is by using fluorescent staining and identify them by flow cytometry, which is presented below.

Fluorescence microscopy and flow cytometry

Several kinds of fluorescent stainings are used to analyse different sperm features. Morphology and membrane integrity, as mentioned above, are some of them. The proportion of live and dead sperm cells, function of mitochondria, acrosomal integrity, membrane integrity, capacitation status and DNA content are some other traits that can be measured using these methods. Different stains are used to detect different parts and different traits of the sperm cells (Gillian *et al.*, 2005; Colenbrander *et al.,* 2003; Love, 2005). Fluorescent sperm cells can be evaluated by fluorescence microscopy or by flow cytometry. Fluorescence microscopy is carried out in a specialized microscope, equipped with a mercury lamp and appropriate filters for selection of light from the different probes.

During flow cytometry the sperm cells flow through a laser beam which causes the cells to fluoresce (Gillian *et al.*, 2005). The light is collected by a photodetector and converted into electronic signals which are stored and displayed by a computer (Sieme, 2009). With computer assistance the sperm cells can be evaluated graphically (Gillian *et al.*, 2005). Flow cytometry is a rapid and usable method for identifying differences that may not be detected by other techniques. A large number of sperm cells can be analysed in a very short period due to a high flow rate. The method also has the capacity to detect several fluorochromes attached to the spermatozoa at the same time, which imply that muliple sperm cells traits can be assessed all together. The disadvantages of this method are the costs, the need for experienced technicians and that the apparatus is sensitive and requires space. Since the method is

expensive it is only utilized in specialized labs (Sieme, 2009). Flow-cytometry performed with suitable stains can offer a significant prediction of fertility in stallions (Colenbrander *et al.*, 2003).

The major components of spermatozoal chromatin are DNA and histones (Griffiths *et al.,* 2008). It is of great importance that the DNA is maintained intact to enable fertilization and normal embryo development. The sperm chromatin structure assay (SCSA) is a method to measure the ability of sperm DNA to maintain its double stranded form (Evenson *et al*., 1980). The results are measured and the influence on fertility is estimated. Normally, DNA of sperm that have been collected, processed and handled properly should not show any signs of denaturation or becoming single-stranded after 46 h of storage. The SCSA is a useful method for raw, cooled and frozen semen to identify a possible reduction in sperm quality. It is an additional assay to evaluate sperm quality and is not routinely done. The technique can also measure variation in DNA quality due to environmental changes, such as the effect of storage time and extender type. These measurements can lead to future improvements in these areas (Love, 2005). Evenson & Lorna (2000) found that SCSA could be useful tool for fertility assessment. Sperm morphology and sperm cromatin structure seems to be interconnected and related to pregnancy rates according to a study presented by Morrell *et al.* (2008).

NucleoCounter

A new technique is the NucleoCounter, which is based on the same principles as the method of fluorescence microscopy. The sperm cells are stained with the fluorescent dye propidium iodide, which binds to the DNA in the cell nuclei. The sample is analysed with a microscope connected to a camera and an image analyser. Signals from the fluorescent dye are detected by the microscope. The system is developed to assisst the production of doses for AI by counting the number of sperm cells in the semen and determines their viability. According to the producer of this equipment, the counting of cells is fast, precise and objective (ChemoMetec, 2009). Some studies have been made to evaluate the method, Hansen *et al.* (2006) found that the method had a high repetability for counting of boar semen. According to Shah *et al.* (2006) the technique also semes to be rapid and have high repetability. The results in this study were also comparable with resluts obtained by standards methods used in semen. Junttila (2009) made a study on stallion semen and found that the NucleoCounter was very easy to use and the analysis was made quickly. The disadvantages were the prize of the equipment and the material needed for the utilization. The results from this study also indicate that the subjective (manual) motility can be replaced by the viability measurements from this technique. This would lead to an increased reliability since this method does not rely on the individual technician in the same way as subjective motility does.

Hypo-osmotic swelling test

Another technique to evaluate membrane integrity is hypo-osmotic swelling test (HOST). A hypo-osmotic solution is used to measure the amount of fluid transported into the spermatozoa across the plasma membrane (Foxcroft *et al*., 2008). Osmotic equilibrium is strived for between the sperm cell and the extracellular environment. Sperm cells with functional membrane will get a coiled tail, caused by swelling and increase in volume to establish equilibrium. Sperm with non-functional membrane will have straight tails which makes them noticeable. The evaluation is carried out with a microscope (Estrada & Samper, 2007).

Longevity assay

The longevity assay is important for semen intended to be cooled and shipped. By knowing longevity of the sperm cells the time that semen can withstand cooling and still be used for successful AI can be predicted. The method is carried out by examination of sperm cell motility in diluted semen after 6, 12, 24 and 48 hours with a microscope (Estrada & Samper, 2007).

Computer-assisted semen analysis

Systems have been developed that makes it possible to analyse semen with computer assistance, as mentioned above. This computer-assisted semen analyses (CASA) is considered a useful tool to analyse several traits such as sperm concentration and motility of sperm cells. The newest models are also able to evaluate sperm morphology. The results are considered more reliable and repeatable then the findings from classical microscopic assessment (Sieme, 2009). For example motility can be evaluated with images equal to the ones used for the track motility technique. The tracks of the motile and the non-motile sperm are recorded and registered by image processing software and then evaluated (Rouge, 2004). The recording of data when using CASA makes it possible to match the results with standard values for comparison. The method can be used both for raw semen and after different treatments such as cooling, freezing etc. The main problem of this technology is that standardization is needed both for the equipment and the procedures. If these problems can be overcome CASA can provide an efficient, precise and reliable tool to improve artificial reproduction technologies, develop physiological understanding and evaluate fertility (Verstegen *et al.*, 2002).

Qualisperm

A newly developed method within CASA systems is Qualisperm. It can be used to analyse concentration, motility and morphology of spermatozoa in semen. This is done by measuring the number of sperm cells crossing a viewable field divided into several detection areas. The movement of the particles, in this case sperm cells, is analysed by an algoritm. The fluctuation signal is caused by light generated from the particles (Tejerina *et al.,* 2008). This is a method that is very accurate, repeatable and easy to use according to the manufacturer. They also state that qualisperm is more rapid than any other CASA system (Biophos, 2009). Evaluation of the system has been made on boar semen and it has been shown that Qualisperm can analyse thousands of spermatozoa per sample, which is a bigger amount then many other methods can analyse. It also provides information about both percentage of motilie sperm cells and their mean speed. These two traits are of big importance when evaluating for AI. For this reason Qualisperm could be a suitable method to use in a routine evaluation of semen (Tejerina *et al.*, 2008).

Density gradient and single layer centrifugation

One way to improve semen quality and increase the fertility rate is to select spermatozoa that are alive and of high quality before insemination. Density gradient centrifugation or the simplified, single layer centrifugation technique, are preparation techniques used for this purpose. Original raw semen includes dead, living and abnormal sperm cells. One way to pick out the most active and motile sperm cells is by using a centrifugation technique. The semen is forced by centrifugation though one (single layer centrifugation) or several (density gradient centrifugation) layers. Only motile sperm cells with normal morphology and intact DNA can pass through. Those sperm cells will be included in the pellet after the procedure.

Dead, abnormal sperm cells and the ones with damaged DNA can not pass through the layers. They will therefore be separated from the high quality sperm cells when removing the supernatant. The result is a pellet of viable cells with normal DNA, called Gradient-prepared spermatozoa. The longevity of these samples is longer than for unprepared samples. It is also free from bacteria and viral infection if prepared carefully. In breeding programs where semen quality is poor, for example in conservation programs or from performance animals, gradient preparation may be a useful tool for selecting high quality spermatozoa from the rest of the ejaculate (Morrell, 2006).

Evaluation of spermatozoa function

In recent years the knowledge of cell function has increased. This expertise can be used for evaluation of sperm cell function in various stages of development and use. There are a number of methods to evaluate the function of spermatozoa (Rodriguez-Martinez, 2003). Stallions with seemingly morphologically normal and motile sperm in their ejaculate may have low fertility. Diagnostic tests for sperm function are therefore needed to determine potential causes and explore possible treatments for this subfertility (Meyers, 2007; Foxcroft *et al.*, 2008).

Acrosomal responsiveness assay

One of the methods for evaluating spermatozoa function is acrosomal responsiveness assay (ARA) which tests the functionality of the spermatozoal acrosome. The assay measures the acrosome ability to react when exposed to a potent inducer, a chemical compund that increase the permability of the membrane to ions, the Ca^{2+} ionophore A23187 (Varner, 2008). The enzymes held by the acrosome are needed during the oozyte penetration as mentioned above (Sjaastad *et al.*, 2003). If the acrosome reaction does not accur when exposed to stimulus the fertility might be influenced (Sieme, 2009). This method can therefore predict fertility of semen containing spermatozoa with acrosomal dysfunction. Morphology or motility of the sperm cell is not affected by this dysfunction, which makes it hard for other techniques to discover it. For example sperm cell with normal acrosomal appearance when using fluorescent light microscopy can have undetected defects (Varner, 2008).

Swim-up

If a medium is placed on top of an ejaculate, motile spermatozoa will swim from the ejaculate into the medium. The sperm cells intend to move away from the seminal plasma and its cellular contents. This technique, called swim-up, is simple to use. An incubator, or at least a water bath, is required so that the semen can be held at body temperature. This is necessary to sustain sperm motility. When the medium is removed from the ejaculate the motile sperm cells will follow, leaving immobile sperm cells, other cells and fragments in the ejaculate. As the separation is based only on motility, morphologically abnormal spermatozoa and spermatozoa with damaged DNA will unfortunately be contained in the medium along with the normal spermatozoa (Morrell, 2006).

Assays for sperm – oocyte interactions

The spermatozoa have to be able to bind to the reached oocyte to achive fertilization and embryo development. There are several assays evaluating different interactions between sperm cells and oocytes. The ability of spermatozoa to penetrate, fuse and bind to the oocyte is analysed. Many of these methods are used in human medicine and have also been

performed in livestock species but are not regularly used in evaluation for stallion semen (Meyers, 2007).

Biochemical markers

Attempts to identify biochemical markers for evaluation of sperm cell function have been made. These markers might be useful in fertility analyses by characterizing enzymatic and functional composition of specific subcellular domains. It is necessary to decide if the enzymes are found only in sperm cells, specific domains of sperm cells, seminal plasma or extender. This can be done by specific markers targeted for a precise goal (Sieme, 2009). Many different methods using biochemial markers have been developed for other species and some have been proposed for stallion spermatozoa. These tests require additional inspection and standardization before further development (Varner, 2008; Sieme, 2009).

Estimation of fertility

Fertilization is influenced by many factors both concerning the male as well as the female. It is a complex process in many steps. The spermatozoa has to be formed, transported and brougt together with the seminal plasma to form the ejaculate. When planted in the female reproductive tract several sperm cell traits have to be sufficient to enable fertilization (Foxcroft *et al.*, 2008).

If semen evaluation is done with the aim to predict fertility, it is necessary to test several parameters (Amann, 1989), since fertility is influenced by so many factors. The quality of the sperm cells and their ability to interact with the oocyte are, as stated before, some of the most impotant traits (Rodriguez-Martinez, 2003). Another feature that has to be taken into consideration is the status of the mare. The oocyte quality, the day for ovaluation and the moment when AI is performed will also affect the result (Bedford-Guaus, 2007). Reasearch in this area have indicated that spermatozoa characteristics seem to be correlated with fertility at a significant level (Amann, 1989) particulary in case of sub-fertility (Morrell *et al.*, 2008). External factors that can affect the result are the inseminator, season and the geografic location (Rodriguez-Martinez, 2003). A good understanding and appropriate handling and processing of the semen before AI are also required (Bedford-Guaus, 2007).

The most accurate way to evaluate stallion fertility is by measuring the outcome of foals in relation to the number of mares mated, as mentioned above. This is a reliable but costly method wich has led to the development of tests measuring fertilization potential of the semen and not only the sperm cell charachteristics (Rodriguez-Martinez, 2003). It has to be taken into accunt that no reliable prediction of stallion fertility can be made out of the pregnancy rate if the fertility of the mares is unknown. It is logical that breeders request information of stallion fertility but it is not logical to expect a similar result if using a mare with no fertility evaluation (Amann, 2006).

Discussion

Spermatozoa are terminal and highly differentiated cells, which have many characteristics needed for fertilization. This makes it impossible to evaluate fertility by only one single test. A combination of tests, measuring one or more traits each, gives a better estimation of fertility than using only one test (Colenbrander *et al.*, 2003; Rodriguez-Martinez, 2003; Estrada & Samper, 2007). When different traits in semen are evaluated, only a small sample from the

semen is used for each test. Sperm cells in semen are heterogenous by nature and this can lead to missleading estimations of sperm quality (Rodriguez-Martinez, 2003).

The aim for present research, in this area, is to develop methods for evaluating as many important spermatozoa trait as possible combined with a quick and inexpensive performence (Colenbrander *et al.*, 2003). An increased knowledge about spermatozoa and the molecular mechanisms that regulate their development and function may lead to new techniques and laboratory tests that can fulfil these requirements. Also therapeutic methods for reducing infertility in stallions may develop. This process will however require much time and funding. Coming generations may also look beyond morphology and motility of spermatozoa when evaluating structual and functional traits and possible defects (Varner, 2008). Some research have lead to the assumption that certain sperm defects are more important to fertility then others but this is not yet completely justified (Sieme, 2009). The center of attention in future research will therefore probably be to decide which combinations of traits that correlates most with fertility. The result might differ if the semen is utilized fresh or after freezing (Colenbrander *et al.*, 2003).

Scientists seem to agree that current laboratoty tests only provide estimation, no prediction, of fertility. To decide if laboratory tests are correlated with fertility, both the test and the documentation of fertility has to be performed with high precision and accuracy to achieve reliable results. This is not easy to do since the tests are performed *in vitro* and only involve some of the factors needed for fertilization *in vivo (*Amann, 1989; Colenbrander *et al.*, 2003; Rodriguez-Martinez, 2003). The tests have to be objective, with as low bias as possible, to obtain reliable results. The most common bias is the human error. The tests also have to be repetable and achieve results that are comparable every time they are used. It also has to be precise and evaluate all sperm characteristics with exactness (Rodriguez-Martinez, 2003). Many current tests are therefore of better use when detecting the causes of potential sub-fertility than for predicting the level of fertility (Colenbrander *et al.*, 2003).

There are several factors that can lead to incorrect estimation of fertility, some of them are insemination of insufficient number of females, too few samples of semen analysed from the male, too big or small amount of spermatozoa used during AI and treatments related to the AI that are unreported or unknown (Amann, 1989). There are also studies showing that fertility is decreased after cooling and transporting spermatozoa for AI (Morrell, 2008). The outcome may also be affected by handling and processing techniques of the semen as well as the use of optimal breeding management strategies (Bedford-Guaua, 2007). All this features have to be taken into consideration when evaluating fertility. The influence of external and internal factors, such as toxin exposure from the environment and the endocrine, autocrine, and paracrine control of spermatozoagenese epididymal dysfunction, has to be further evaluated in the future (Sieme, 2009).

Conclusion

The aim of this literature review was to find out if the most common laboratory tests for sperm quality used to day can be applied to predict fertility in stallions. My conclusion is that further reasearch is needed in this area. I think that a lot of facts, concerning both spermatozoal function and the complex fertilization process, are yet to be discovered. Development of new techniques and deeper evaluations of the present ones are also desired. A greater knowledge in both these matters might in the future lead to an increased

understanding and thereby better, more accurate, laboratory tests. This might enable prediction and not only estimations of fertility in stallions, as well as in other species.

References

Amann, R.P. 1989. Can the Fertility Potential of a Seminal Sample Be Predicted Accurately? Journal of Andrology, Vol. 10, No. 2, 89-98.

Amann, R.P. 2006. The fertility dilemma: perception vs. actuality Equine Veterninary Education 18: 3, 159-164.

ASVH, Avelsföreningen för Svenska Varmblodiga Hästen. Mars 2009. http://asvh.se/document/betackning08.htm

Bedford-Guaus, S. J. 2007. Transported Stallion Semen and Breeding Mares with Cooled or Frozen-Thawed Semen. Clinical Techniques in Equine Practice 6, 239-248.

Biophos. April 2009. http://biophos.com/web/eng/technology.htm

ChemoMetec. April 2009. http://www.chemometec.com/sw3132.asp

Colenbrander, B., Gadella, B.M., Stout, T.A.E. 2003. The Predictive Value of Semen Analysis in the Evaluation of Stallion Fertility. Reproduction in Domestic Animals 38, 305–311.

Estrada, A.J., Samper, J.C. 2007. Evaluation of raw semen. In: Current Therapy in Equine Reproduction (eds. J.C. Samper., J.F. Pycock., A.O. McKinnon), 253-257. Saunders Elsevier, Missouri, USA.

Evenson, D., Lorna, J. 2000. Sperm chromatin structure assay is useful for fertility assessment. Methods in Cell Science 22, 169–189.

Evenson, D.P., Darzynkiewicz, Z., Melamed, M.R. 1980. Relation of Mammalian Sperm Chromatin Heterogeneity to Fertility. Science, New Series, Vol. 210, No. 4474, 1131-1133.

Evenson, D.P., Parks, J.E., Kaproth, M.T., Jost, L.J. 1993. Rapid Determination on Sperm Cell Concentration in Bovine Semen by Flow Cytometry. Journal of Dairy Science 76, 86-94.

Foxcroft, G.R., Dyck, M.K., Ruiz-Sanchez, A., Novak, S., Dixon W.T. 2008. Identifying useable semen. Theriogenology 70, 1324–1336.

Gillan, L., Evans, G., Maxwell, W.M.C. 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 63, 445–457.

Griffith, A.J.F., Wessler, S.R., Lewontin, R.C., Carroll, S.B. 2008. Genes and Chromosomes. In: Introduction to genetic analysis. 31-36. W.H. Freeman and Company, New York, USA.

Hansen, C., Vermeiden, T., Vermeiden, J.P.W., Simmet, C., Day, B.C., Feitsma, H. 2006. Comparison of FACSCount AF system, Improved Neubauer hemocytometer, Corning 254 photometer, SpermVision, UltiMate and NucleoCounter SP-100 for determination of sperm concentration of boar semen. Theriogenology 66, 2188–2194.

Holt, W.V., O'Brien, J., Abaigar, T. 2007. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. Reproduction, Fertility and Development 19, 709–718.

Junttila, L. 2009. Effekt av spädningsvätska på spermiemembranintegritet analyserad med NucleoCounter SP-100. Sveriges lantbruksuniversitet, Fakulteten för veterinärmedicin och husdjursvetenskap, Veterinärprogrammet. Examensarbete 2009:21.

Lärn-Nilsson, J., Jansson, S.D., Strandberg, L. 2005. Naturbrukets Husdjur. Del 1. (eds. Å. Wennström, A.G. Stenung), 57-58. Alfa Print, Sundbyberg, Sweden.

Love, C.C. 2005. The sperm chromatin structure assay: A review of clinical applications. Animal Reproduction Science 89, 39–45.

Meyers, S.A. 2007. Advanced Semen Tests for Stallions. In: Current Therapy in Equine Reproduction (eds. C. Samper., J.F. Pycock., A.O. McKinnon), 275-280. Saunders Elsevier, Missouri, USA.

Morrell, J.M. 2006. Update on Semen Technologies for Animal Breeding. Reproduction in Domestic Animals 41, 63–67.

Morrell, J.M., Johannisson, A., Dalin, A-M., Hammar, L., Sandebert, T., Rodriguez-Martinez, H. 2008. Sperm morphology and chromatin integrity in Swedish warmblood stallions and their relationship to pregnancy rates. Acta Veterinaria Scandinavica 50:2.

O'Connor, M.T., Amann, R.P., Saacke, R.G. 1981. Comparisons of Computer Evaluations of Spermatozoal Motility with Standard Laboratory Tests and their use for Predicting Fertility. Journal of Animal Science 53, 1368-1376.

Rodriguez-Martinez, H. 1996. Reproductive physiology of the stallion. In: Compendium on Equine Reproduction (eds. A-M. Dalin, L. Malmgren), 131-141. SLU, Uppsala, Sweden.

Rodriguez-Martinez, H. 2003. Laboratory Semen Assessment and Prediction of Fertility: still Utopia? Reproduction in Domestic Animals 38, 312-328.

Rouge, M. June 2004. Collection and Evaluation of Semen: Introduction and Index. http://www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneval/motility.html

Shah, D., Naciri, M., Clee, P., Al-Rubeai, M. 2006. NucleoCounter—An efficient technique for the determination of cell number and viability in animalcell culture processes.Cytotechnology 51, 39–44.

Sieme, H. 2009. Semen Evaluation. In: Equine Breeding Management and Artificial Insemination (ed. J.C. Samper), 57-70. Saunders Elsevier, Missouri, USA.

Sjaastad, Ø .V., Hove, K., Sand, O. 2003. Reproduction. In: Physiology of Domestic Animals. (eds. C.Steel, VESO), 621-669. Scandinavian Veterinary press, Oslo.

Söderquist, L. 1996. Artificial Insemination. In: Compendium on Equine Reproduction (eds. A-M. Dalin, L. Malmgren), 172-173. SLU, Uppsala, Sweden.

Tejerina, F., Buranaamnuay, K., Saravia, F., Wallgren, M., Rodriguez-Martinez, H. 2008. Assessment of motility of ejaculated, liquid-stored boar spermatozoa using computerized instruments. Theriogenology 69, 1129–1138.

Varner, D.D. 2008. Developments in stallion semen evaluation. Theriogenology 70, 448–462.

Verstegen, J., Iguer-Ouada, M., Onclin, K. 2002. Computer assisted semen analysers in andology research and veterinary practice. Theriogenology 57, 149-l79.