GRANSKAD ARTIKEL

SPOTLIGHT ON SEMEN QUALITY FOR EQUINE ARTIFICIAL INSEMINATION

Equine artificial insemination is very common in Sweden, with the vast majority (more than 97 %) of warmblood brood mares currently being bred by this method. Cooled semen is the most frequently used material although frozen semen is slowly gaining in popularity, particularly for trotters. A breakdown of the different sorts of semen used is given in Figure 1, compiled by Professor A-M. Dalin from figures provided by the Association for Warmblood trotters and riding horses, respectively. The proportion of mares bred by natural mating was 3 % for warmblood trotters and 0.5 % for warmblood riding horses

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Foaling rates after AI with the different types of semen vary slightly with the type of semen used, as shown in Table 2 for mares foaling during 2018 (i.e. inseminated during 2017).

A successful outcome after artificial insemination depends on many factors; among them sperm quality and microbial contamination are two important aspects. Research by the author and by two PhD students at the Swedish University of Agricultural Sciences (SLU) has focused on these topics.

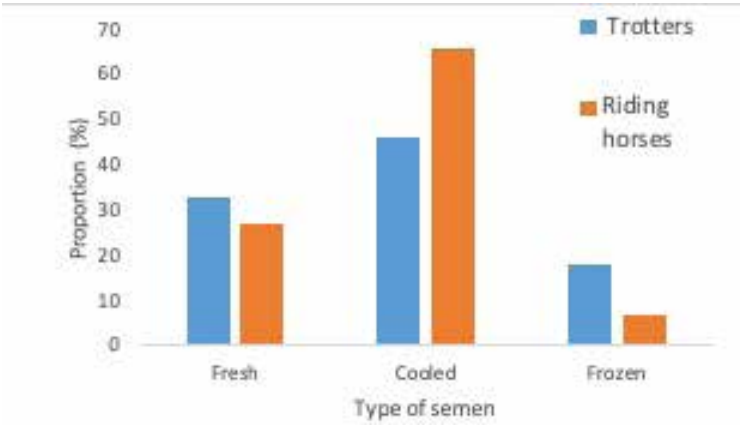

Figure 1: types of semen used for equine artificial insemination in Sweden in 2018. (Compiled by Professor Anne-Marie Dalin at the Swedish University of Agricultural Sciences)

Figure 2: foaling rates according to type of semen used for equine artificial insemination in 2017. (Compiled by Professor Anne-Marie Dalin at the Swedish University of Agricultural Sciences)

Can we improve stallion sperm quality in semen doses for artificial insemination?

The ejaculate contains a heterogeneous mixture of spermatozoa of all ages and stages of maturity, only some of which will be capable of eventually fertilizing an oocyte. The possibility of selecting such spermatozoa so that only potentially "useful" ones are inseminated could help to improve foaling rates. This was the theory behind a series of experiments carried out by Professor Jane Morrell and colleagues at the Swedish University of Agricultural Sciences. She found that centrifuging the spermatozoa through a colloid containing silane–coated silica particles (Single Layer Centrifugation; SLC) enabled the most robust spermatozoa to pass to the bottom of the tube (Morrell & Nunes, 2018). These were typically spermatozoa with normal morphology, intact membranes and unfragmented DNA. These spermatozoa live

much longer than unselected spermatozoa when cooled – at least 96 hours after semen collection – and fertilization rates were still high at this time in a small insemination trial (Lindahl et al., 2012). This led to a controlled insemination trial on a large number of mares, involving studs in three countries (Morrell et al., 2014a). Here ejaculates were split, with one part being prepared by SLC and the other part serving as the control. The sperm samples were cooled and stored for 24 h before insemination. The mares were examined with ultrasound 14-16 days after insemination; an embryonic vesicle was found in 69% mares that had received SLC-selected sperm doses compared to 42% that received the control doses ($P<0.01$).

Apart from using SLC to improve sperm quality, both for "problem" stallions and for "normal" stallions, being able to extend the "shelf-life" of sperm doses is an interesting prospect. By convention, semen is collected three times a week a most studs in Sweden, and transported to other studs for insemination. This means that cooled semen doses may not be available just when the mare is ready to ovulate; it is a particular problem at the weekend or during public holidays. Knowing that SLC-selected sperm doses have good fertilizing capacity after 96 h could make ordering of semen doses and planning of inseminations much easier.

How does the technique work? Spermatozoa are damaged by byproducts of cell metabolism called "reactive oxygen species" e.g. hydrogen peroxide. Damaged cells also act a source of more reactive oxygen species, leading to a cascade effect within the sperm sample. Storing sperm doses during transport increases the likelihood that normal spermatozoa will be damaged by these metabolic byproducts so that they will be unable to function after insemination. Separating the most robust spermatozoa from dead spermatozoa and other cells, as well as from seminal plasma, enables them to be maintained in a healthy condition for longer than in unselected samples.

An intriguing fact about SLC-selected stallion spermatozoa is that they produce practically no hydrogen peroxide, whereas their production of superoxide (another type of reactive oxygen species) is increased (Morrell et al., 2017). Superoxide does not appear to be harmful to other spermatozoa, unlike hydrogen peroxide. An interpretation of this observation could be that the selected spermatozoa are metabolising more, or in a different way, to the unselected sperm populations. The number of these selected spermatozoa can also be used as an indicator of the likely fertility of the stallion. Thus the "recovery rate" or how many of the spermatozoa from the original samples pass through the colloid, can be used as an indicator of the stallion's fertility (Morrell et al., 2014b). Such a test could help to identify stallions that have a fertility problem at an early stage, before a large number of mares have been inseminated and subsequently found not to be pregnant.

For a review of the uses of SLC to prepare stallion semen for equine artificial insemination, see Morrell (2011).

Can manipulating seminal plasma improve stallion sperm cryosurvival?

This project was undertaken by PhD student Essraa Al-Essawe, in conjunction with Professor Jane Morrell and Professor Anders Johannisson at SLU. Much research has been done with freezing stallion spermatozoa but it is still not possible to freeze all ejaculates successfully. This has led to the classification of stallions as being "good" or "bad" freezers, based on post-thaw sperm motility. Even if post-thaw sperm motility is deemed to be acceptable, the pregnancy rates after artificial insemination with frozen semen for some stallions tend to be lower than with liquid semen, necessitating careful timing of insemination as close to ovulation as possible.

One suggestion to improve sperm cryosurvival is to select the most motile, viable spermatozoa with good morphology and chromatin integrity by SLC prior to cryopreservation, on the basis that these spermatozoa have the best chance of surviving freezing and thawing, if sources of reactive oxygen species have been removed. Initial results with SLC prior to cryopreservation showed that post-thaw sperm quality could be improved (Hoogewijs et al., 2011) and the length of post-thaw sperm survival was extended (Hoogewijs et al., 2012). It was also possible to use SLC post-thawing.

Previous research on pig spermatozoa found that the inclusion of small proportions of seminal plasma with the sperm suspension may be beneficial by helping to repair membrane damage induced by cryopreservation. However, similar studies with stallion spermatozoa produced conflicting results. These differing results may be due to the difficulties in removing all seminal plasma, and thus not being able to control the proportion of seminal plasma in the "treated" sperm samples. Since all of the seminal plasma can be removed from the spermatozoa with SLC (Kruse et al., 2011), a PhD project was devised in which known quantities of seminal plasma were added to SLC-selected (seminal plasma–free) sperm samples, either before freezing or after thawing. Essraa Al-Essawe evaluated sperm quality in the treated samples and controls (SLC only). She found that sperm quality was improved in the SLC samples but that adding seminal plasma did not provide an additional benefit. However, there were differences in the effect of seminal plasma from "good" and "bad" freezer stallions, in that adding seminal plasma from a bad freezer stallion resulted in greater amounts of hydrogen peroxide being produced (Al-Essawe et al., 2018a). This metabolic byproduct causes damage to spermatozoa. Adding the seminal plasma after thawing did not repair damaged membranes although the addition of SP from good freezers stallions did increase sperm metabolic activity whereas SP from bad freezer stallions did not (Al-Essawe et al., 2018b).

Evaluating sperm quality indirectly can be a useful indicator of the potential fertilizing ability of the sample but does not show that the spermatozoa can actually fertilize oocytes. Fertility trials are difficult in horses because finding enough mares to conduct an artificial

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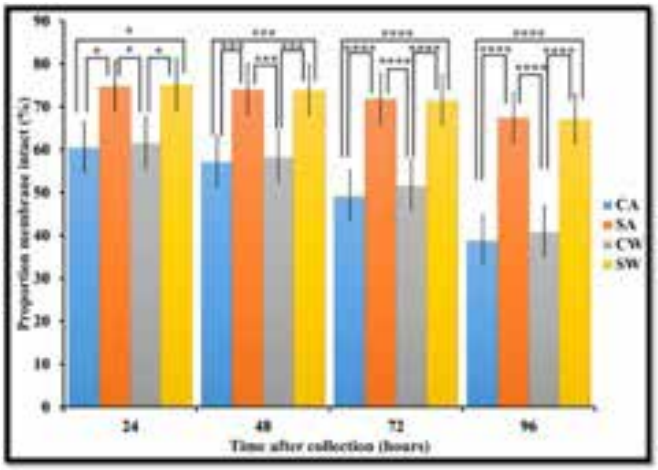

Figure 3: Sperm viability in SLC and control sperm samples, with or without antibiotics (n=18) (from Al-Kass et al., Antibiotics 2018:7, 1).

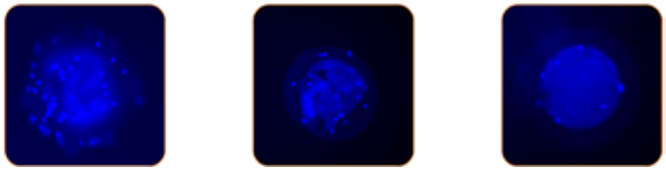

Figure 4: stallion spermatozoa bound to bovine oocytes in a laboratory test for fertilizing ability.

insemination trial is impractical and it has not been possible to develop in vitro fertilization system that is reliable in this species. Essraa investigated the ability of stallion spermatozoa to bind to bovine oocytes in a heterologous binding assay. She observed that adding seminal plasma increased the number of spermatozoa binding, depending on when the seminal plasma was added (before freezing or after thawing) and whether the seminal plasma came from good or bad freezer stallions (Al-Essawe et al., 2018c). These results suggest that adding seminal plasma to thawed spermatozoa before insemination could be used to increase the time for which they remain functional in the mare, thus making the timing of insemination relative to ovulation less critical than with untreated spermatozoa. Insemination trials are now needed to test this hypothesis.

Can we remove bacteria from semen samples as an alternative to using antibiotics?

The lower part of the reproductive tract is contaminated by bacteria from the skin and the environment, and these bacteria are transferred to the semen during ejaculation. Further contamination can occur from the environment during processing of the semen doses. Some of these bacteria can potentially cause disease in inseminated mares, for example Taylorella equigenitalis, Klebsiella pneumoniae, Pseudomonas spp. and beta haemolytic streptococci. Even if bacteria do not cause disease, they may have an adverse effect on sperm quality, resulting in decreased fertility and a reduction in the length of time for which the spermatozoa will remain viable. Antibiotics are added to semen extenders to control the growth of these bacteria but it is not known whether such non-therapeutic use could be contributing to the development of antibiotic resistance.

Ziyad Al-Kass investigated the use of a modified SLC to remove bacteria from stallion semen in his PhD project on characterization, quantification and removal of potential pathogens from stallion semen. In this project, he identified the bacteria commonly occurring in semen from Swedish stallions and evaluated what proportion of these bacteria could be removed. In addition, he stored the sperm samples in semen extender with or without antibiotics and analysed sperm viability and DNA integrity, amongst other properties. He found that the modified SLC samples remained viable for longer than control samples, in agreement with previous studies. There was a considerable reduction in the number of bacteria in the SLC samples. The total bacterial counts were as follows: control with antibiotics 1x106, SLC with antibiotics 0,2 x106, control without antibiotics 4,6 x106 and SLC without antibiotics 0,8 x106. Thus modified SLC reduced the bacterial load to 18 -25% in the presence or absence of antibiotics, respectively. Surprisingly, although modified SLC improved sperm quality, adding antibiotics did not provide an additional benefit, implying that the bacteria found in these semen samples did not have a detrimental effect on sperm quality. Overall, the results of this experiment showed that modified SLC could reduce the bacterial contamination in semen.

Conclusions

The results of these experiment show that SLC is a useful tool for stallion semen preparation. It involves a 20 minute centrifugation and can be carried out easily at the stud providing there is access to a centrifuge. The extra time involved in preparation can be easily justified in better sperm quality in the selected sperm samples and improved pregnancy rates or cryosurvival. The ability to separate spermatozoa from most of the bacteria found in semen samples could enable a reduction in the antibiotics used in semen extenders, which in turn would help the fight against the development of antibiotic resistance. •

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