Improving cryosurvival of stallion spermatozoa for artificial insemination

In December 2014, The Foundation for Equine Research (Stiftelsen Hästforskning) granted funding (2 100 000 SEK) for a project to improve cryosurvival of stallion spermatozoa. This report provides an account of the major findings arising from this study. Please note that in this report, citations of previously published work are denoted by [n], whereas publications arising from this project and manuscripts in preparation are indicated by (***authors, date***).

Objectives: The project had two main objectives:

1. To determine the role of seminal plasma in stallion sperm cryosurvival and sperm quality;
2. To investigate the effects of adding components of reproductive tract secretions (heat shock proteins and prostasomes), to stallion sperm samples, to increase cryosurvival and survival *in vivo*.

Methods

Our hypothesis was that incorporating seminal plasma (SP), heat shock proteins (HSP) and/or prostasomes in handling protocols for stallion semen would improve sperm cryosurvival. In the first work package, the effect of adding SP from stallions of known fertility to SP-free stallion sperm samples was studied. Thus the stallions were designated as either "good freezers" (GF) or "poor freezers" (PF) on the basis of historical test freezing results. The SP was added either before freezing or after thawing. Sperm functional activity was assessed using a heterologous zona binding assay, since equine *in vitro* fertilization cannot be done reliably or repeatably. In the second work package, the effects of adding HSP and/or prostasomes to stallion spermatozoa was studied. Heat shock protein HSP8, previously used with bull and boar spermatozoa [1], was kindly made available by Dr A. Fazelli, University of Sheffield.

Work package 1: Effects of seminal plasma

Experiment 1: Pooled SP was prepared from stallions of known freezing ability (6xGF and 6xPF). Small quantities of these pooled SP samples were added to SP-free spermatozoa from other stallions, i.e. where the SP has been removed by colloid centrifugation, using 3 ejaculates per stallion. A cross-over design was used, as follows:

i. Spermatozoa from good freezer + good seminal plasma
ii. Spermatozoa from good freezer + poor seminal plasma
iii. Spermatozoa from poor freezer + good seminal plasma
iv. Spermatozoa from poor freezer + poor seminal plasma

The treated sperm samples were frozen and post-thaw sperm quality was determined compared to controls, which retained their own SP. Sperm motility was assessed with Computer Assisted Sperm Analysis [2]; flow cytometric assessments of membrane integrity [3], chromatin integrity [4], ROS-content [5], mitochondrial membrane potential [6] were made, after staining with the appropriate fluorophores.

Experiment 2: Known amounts of pooled SP from GF or PF was added to SP-free sperm samples after thawing. Sperm quality was evaluated as for Experiment 1.

Experiment 3: The effect of seminal plasma on sperm function was investigated using a heterologous zona binding assay [7]. The intention was to develop an assay for stallion spermatozoa to test whether the addition of seminal plasma had an effect on the ability of the spermatozoa to bind to bovine oocytes.

Single Layer Centrifugation (SLC): SLC was performed by layering 15 mL extended semen on 15 mL Equicoll. The preparation was centrifuged at 300 x ***g*** for 20 minutes, after which the supernatant and most of the colloid was discarded. The sperm pellet was resuspended in semen extender and was adjusted to the appropriate sperm concentration [8].

Sperm quality: Sperm motility was analysed by computerized sperm motility analysis (CASA) for sperm motility and kinematics [8]. Sperm viability was evaluated by staining with SYBR-14/propidium iodide and analysing by flow cytometry. This method permits a large number of spermatozoa to be analysed in a short period of time and improves objectivity compared to the use of fluorescence microscopy [3]. For sperm chromatin integrity, the Sperm Chromatin Structure Assay [4], slightly modified for stallion sperm samples [3], was used which involved staining the sperm chromatin with acridine orange and analysing the resulting fluorescence by flow cytometry. Production of reactive oxygen species was determined by staining sperm samples with hydroethidine and dichlorodifluoro-acetate to determine superoxide and hydrogen peroxide content by flow cytometry [5]. Mitochondrial membrane potential was measured by staining with JC-1 and measuring the resulting fluorescence by flow cytometry [6]. For some experiments, staining with JC-1 and MitoSox was performed in order to detect mitochondrial activity and superoxide production simultaneously.

Sperm cryopreservation: the standard technique available at the stud was used [9].

Heterologous zona binding assay: the method of Macías García [7] was used.

Work package 2: composition of seminal plasma

The proportions of heparin-binding proteins (fertility-associated proteins) present in seminal plasma of GF and PF stallions in the breeding season and non-breeding season were studied using Fast Protein Liquid Chromatography. In addition, the protein composition of seminal plasma from gF and PF stallions was studied by mass spectrometry and Western blotting, in a collaboration with researchers at the French National Institute for Agricultural Research (INRA).

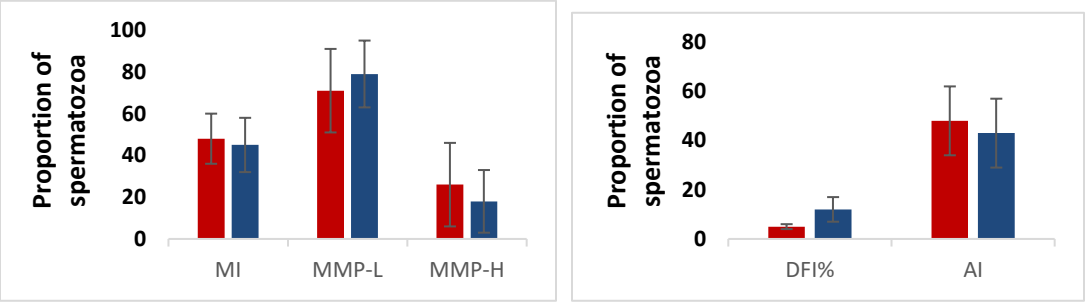
Work package 3: effects of adding heat shock proteins or prostasomes

Additives such as HSP or prostasomes were added to SP-free sperm preparations (prepared by SLC). Sperm motility and other parameters of sperm quality were assessed by CASA and flow cytometry as described above.

Results

Effect of seminal plasma from good and poor freezers (GF and PF, respectively) on post-thaw sperm quality in samples prepared by Single Layer Centrifugation. Sperm motility did not differ between treatments (***Al-Essawe et al., 2018a***). However, there were differences between the controls and SLC in membrane integrity (P<0.05), mitochondrial membrane potential (P<0.001), chromatin integrity (P<0.001), and in the proportion of live sperm with non-reacted acrosome (p<0.001) (Figure 1). The SLC samples had better sperm quality than controls, regardless of whether the spermatozoa came from good or poor freezers (Table 1). Adding SP post-thaw did not repair cryoinjuries (***Al Essawe et al., 2018b***).

Figure 1: Effect of SLC on a) sperm membrane integrity and mitochondrial membrane potential; b) %DFI and acrosome integrity (mean ± SEM)



Note: red bars represent SLC samples, blue bars controls. MI = Membrane integrity, MMP = mitochondrial membrane potential, (-H, -L = high, low), %DFI = DNA fragmentation index, AI = acrosome integrity.

Table 1: Effect of SLC on sperm quality and freezability, according to source of spermatozoa

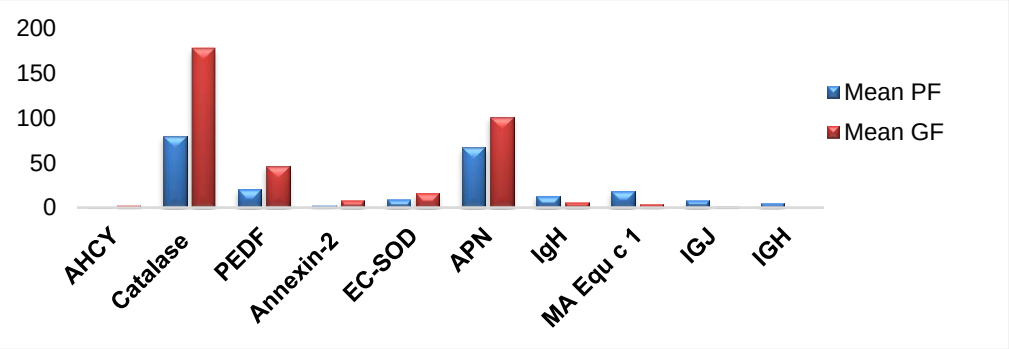
Groups	Total motility%	Intact plasma membranes	%DFI	Non-active mitochondria	Active mitochondria
Control spz from GF	57±10[a]	45±15	11±5[b]	78±16[d]	19±15[d]
SLC, spz from GF	59±18	50±13	5±3[b]	72±20[d]	26±20[d]
Control spz from PF	44±23[a]	45±12	13±5[c]	80±15[e]	17±15[e]
SLC, spz from PF	48±27	47±12	6±2[c]	70±20[e]	27±20[e]

Note: spz = spermatozoa; GF = Good Freezers; PF = Poor Freezers. %DFI = DMA fragmentation index. Same superscripts within a column denote statistical significance. a = p<0.05; b and c = P<0.001; d and e p<0.01.

Differences in composition of seminal plasma between good and poor freezers

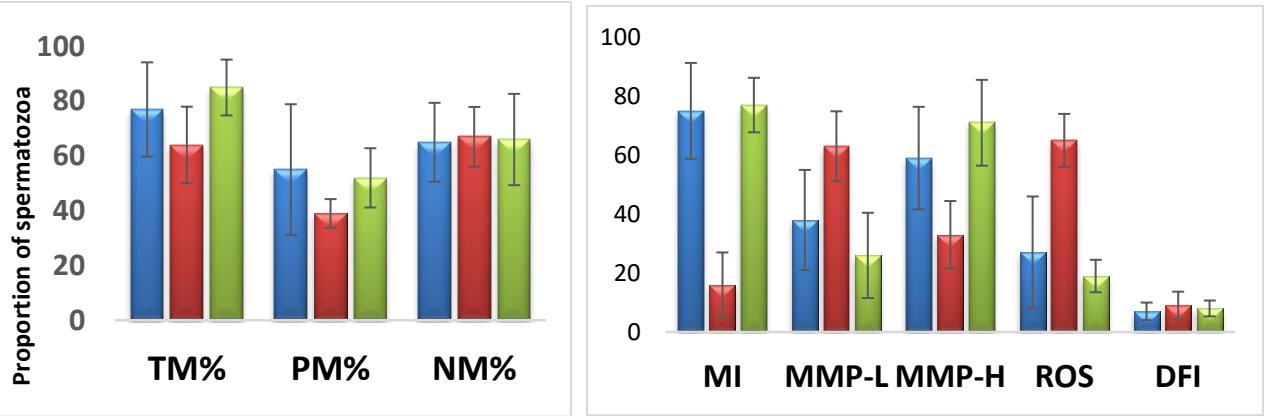
Mass spectrometry was carried out on pooled seminal plasma from good and poor freezers (GF and PF, respectively). Differences were observed in the levels of 33 proteins between the two groups ($p < 0.05$ to $p < 0.001$, depending on the protein) (Figure 2). Pooled GF had high levels of antioxidants (e.g. catalase and superoxide dismutase) and motility stimulants e.g. aminopeptidase N, whereas pooled PF had high levels of immunoglobulins and associated proteins. These proteins have been characterized further, in collaboration with a group in France (***Al Essawe et al., in preparation***).

Figure 2: Total normalised spectra (arbitrary units) of seminal plasma proteins in Good Freezer (GF) versus Poor Freezer (PF) stallions



An unexpected result was found in the analysis of the fresh semen from the ejaculates that were collected to harvest seminal plasma. In our samples, sperm quality during the breeding season was lower than in the non-breeding season (Figure 3). This observation may reflect seasonal changes in SP.

Figure 3: Effect of season on "poor cooler" stallion sperm quality (mean ± SEM).

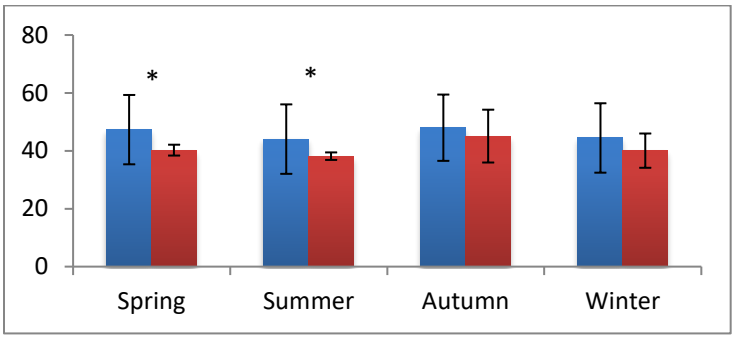


Note: Blue bars = February (non-breeding season), red bars = May (breeding season) green bars = November (non-breeding season). TM = total motility, PM = progressive motility, NM = normal morphology, MI = membrane integrity, MMP = mitochondrial membrane potential, -L, -H = low, high, ROS = reactive oxygen species, DFI= DNA fragmentation index.

Some of these stallions (n=3) were known to be "poor coolers" in that their spermatozoa did not survive well in **cooled** semen doses unless most of the seminal plasma was removed, but to our knowledge this is the first time that differences in the seminal plasma protein composition of such stallions between the breeding season and non-breeding season have been reported.

Seasonal changes in types of proteins in good and poor freezer stallions: Phosphorylcholine-binding proteins were the most abundant category in samples from both good and bad freezers. There was a significant difference in the ratio of non-heparin binding proteins to total proteins (Figure 4) between good and bad freezers in spring (p=0.030) and summer (p=0.048) (***Johannisson et al., in preparation, a***).

Figure 4: proportion of non heparin-binding proteins among total proteins (%) for good and poor freezer stallions in different seasons.



Note: * denotes significant difference between good freezers (blue bars) and poor freezers (red bars).

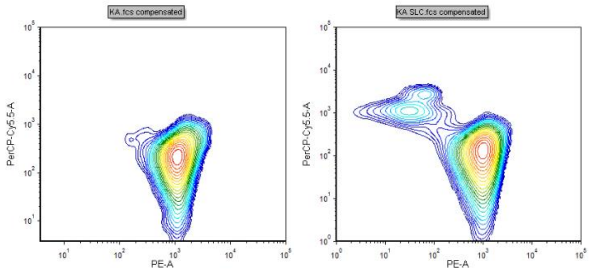
Effect of Single Layer Centrifugation on sperm sub-populations

During the investigations of metabolic activity in spermatozoa, especially in SLC-selected samples, an enrichment for a sperm sub-population with high superoxide production and low mitochondrial membrane potential was observed in the fresh SLC samples (Figure 5). Normally one would expect that spermatozoa with low mitochondrial potential would have a low production of superoxide (a metabolic byproduct) (***Johannisson et al. in preparation, b***). Therefore, the appearance of this sub-population with high superoxide production and low mitochondrial potential is interesting. Since SLC-samples are highly fertile and retain their fertility for at least 96h [10][11], it is interesting to speculate about the function of this particular sub-population. We speculate that this sperm sub-population is capable of surviving for a long time in the female reproductive tract by conserving energy stores but at the same time initiating capacitation to be able to respond when ovulation has occurred. Investigations are continuing.

Studies comparing SLC and sperm washing (a method of removing seminal plasma commonly used to prepare cooled semen doses in other countries, e.g. Germany) showed that the SLC-selected donkey sperm samples had better sperm quality than those prepared by sperm washing (Ortiz et al. 2015), and

SLC-selected stallion sperm samples survived storage better than sperm washing, although there was an interaction with the extender used (***Richter et al., in preparation***).

Figure 5: Stallion spermatozoa simultaneously stained for superoxide production and mitochondrial membrane potential: control (left) and SLC-selected samples (right).



Heterologous zona binding assay

There were differences in the number of stallion spermatozoa binding to bovine oocytes depending on the type of SP added to the sample and whether it was added before freezing or after thawing. Adding SP from GF stallions after thawing prior to filtration by low-density SLC increased binding to oocytes whereas SP from PF stallions did not. An interesting effect was that SP from good freezer stallions, added prior to cryopreservation, reduced binding to bovine oocytes (***Al Essawe et al., 2018c***).

Heat shock proteins/prostasomes

Adding heat shock proteins to stallion sperm samples did not produce a measurable effect on sperm quality. There were some problems with the production of sperm-free prostasome samples, and we are waiting for a new rotor for the ultracentrifuge before continuing with these experiments.

Conclusions

The ability of stallion sperm to withstand cryopreservation is related to components of SP, which in turn is affected by season. The initial exposure of sperm to antioxidants in SP may protect the sperm during subsequent cryopreservation, even though most of the SP is removed during processing. The effect of SP from GF stallions can be beneficial, depending on when it is added i.e. prior to freezing or after thaing; therefore, insemination protocols for SP-treated sperm may require adjusting, particularly concerning the timing of insemination relative to ovulation, depending on the origin of the SP. These results will be used to optimise sperm handling procedures.

Significance to the equine industry

Frozen semen is used infrequently for artificial insemination in Sweden. Among Standardbred trotters and Swedish Warmblood riding horses, the proportions were 19% and 7%, respectively, in 2017, out of 3708 and 4374 mares inseminated. The reasons for this low usage are partly because of the perception that AI with frozen semen is more demanding and less successful than AI with fresh or cooled semen due to the need to carry out the insemination close to ovulation, and partly due to the low

success rate in freezing ejaculates from some stallions. Some studs have a freezing programme for their most popular stallions but improved methods for freezing, or better cryosurvival, could lead to a substantial increase in the use of frozen semen. The equine breeding industry in Sweden has itself expressed considerable interest in being able to improve the quality of frozen sperm doses for AI, both to increase pregnancy rates on studs throughout the country and to improve the reputation and competitiveness of the national semen industry globally. The quality and usage of frozen semen should be improved in order to enhance competitiveness in global markets.

The addition of seminal plasma to sperm samples before or after freezing is a relatively easy procedure that would be accessible to all studs where there is a centrifuge. However, the results from this study indicate that removing seminal plasma by colloid centrifugation is beneficial to sperm quality, whereas adding seminal plasma from good freezer stallions does not provide an additional benefit and seminal plasma from poor freezer stallions can be detrimental. The ability of the spermatozoa to bind to bovine oocytes was affected by the addition of seminal plasma from good freezer stallions, since fewer spermatozoa were observed to be attached to the zona pellucida. This observation could reflect an increased level of decapacitation factors in the seminal plasma of good freezer stallions, although this hypothesis was not tested in our study. For practical purposes, it could mean that the timing of insemination of treated spermatozoa could be earlier than is currently done, since the spermatozoa take longer to capacitate to be ready for fertilization than in untreated samples. However, the validity of this theory should be tested in artificial insemination trials before recommendations can be made.

An interesting, previously unreported, seasonal effect on seminal plasma proteins was observed in our study, causing a detrimental effect on sperm survival during cooled storage during the breeding season. It is already known that some semen samples survive better if most of the seminal plasma is removed, but our study provided clear evidence that it the protein composition of seminal plasma may be responsible for this effect. This result suggests that many semen samples could benefit from preparation by colloid centrifugation, since this method allows the seminal plasma to be removed without having a detrimental effect on chromatin integrity. However, this was not the main focus of the present study, being a side issue arising from the analysis of proteins in seminal plasma.

In addition, many equine breeds are considered to be rare or even endangered, due to depletion in the numbers of breeding animals. Effective conservation of rare equine breeds would be greatly facilitated by the availability of stocks of cryopreserved semen, not only by preserving valuable genetic material when a stallion dies or is castrated, but also facilitating distribution of semen. Furthermore, frozen semen can be “quarantined” until it has been shown to be free of infectious diseases, e.g. equine arteritis virus, which would thus also benefit animal health and welfare. The use of colloid centrifugation to select the most robust spermatozoa and remove most of the damaged ones before cryopreservation has a positive effect on sperm cryosurvival. The ability to test sperm function by carrying out an *in vitro* binding assay, as described here, would be of considerable benefit, since only those sperm samples that were shown to have retained their fertilizing ability could be stored for future use. These improvements in semen cryopreservation would also benefit the equine breeding industry as

a whole, although it should be noted that standardbred trotters cannot be registered in Sweden if the sire was castrated or died more than one year before the dam was inseminated.

This project has given us the opportunity to study existing problems from a new perspective and to open up new avenues of research, especially with colleagues in different countries (Austria, Germany, Spain, Portugal, Italy, Norway, the United Kingdom and the United States). The results of these studies have considerable practical implications and will be used to update recommendations for methods in equine breeding for studs, breed societies and others interested in rare breed conservation.

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Student projects

Maria Ines Figeuiredo: Changes in stallion sperm mitochondrial membrane potential during storage.

Alexander Brown: Sperm mitochondrial membrane potential and reactive oxygen species production in SLC-selected stallion sperm samples.

Richter J, Stuhtmann G, Martinsson G, Sieme H, Morrell JM. Stallion sperm quality is higher after colloid centrifugation compared to sperm washing, or extension only, in INRA82 or INRA96.

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Al-Essawe et al. Stallion spermatozoa and seminal plasma proteomes: their impact on cryopreservation.

Johannisson et al. Differences in sperm quality, testosterone levels and protein composition of stallion seminal plasma depending on season in good and bad freezers.

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