Project H0747189: Reactive oxygen species in stallion semen: their occurrence, effect on spermatozoa and potential means of control.

Background

Reactive oxygen species (ROS) in semen are produced by dead and damaged spermatozoa, and cytoplasmic remnants in immature spermatozoa, as well as leukocytes and cellular debris. High levels of ROS are associated with infertility in humans (Aitken & Clarkson, 1987), although a low level of ROS is considered to be a prerequisite for the sperm membrane changes occurring prior to fertilisation (Aurich et al, 2005; Morte et al, 2007). In contrast, fertilization in porcine IVF may be improved by reducing ROS levels with superoxide dismutase or catalase (Roca et al, 2005).

Cooled, extended stallion semen, transported overnight in styroform boxes, has produced very variable results in AI. There is great variation between stallions and between ejaculates in the time that spermatozoa retain their fertilising capacity and viability, varying from one hour to 2-3 days. The reasons for this variation have not been identified. A possible contribution to sperm damage by ROS was previously discounted by Kankofer et al. (2005), who considered that the anti-oxidative systems glutathione peroxidase, superoxide dismutase and catalase present in stallion seminal plasma would neutralise ROS. Furthermore, semen extenders may have antioxidative activity: for example, milk, a major component of many extenders used for stallion spermatozoa, is believed to have good anti-oxidative properties. However, extraneous factors may influence ROS production by individual stallions, thereby exceeding the neutralizing capacity of the antioxidants and causing the considerable variability in stallion sperm survival seen during storage.

For stallion spermatozoa, no universally suitable cryopreservation method has been developed yet. Ejaculates from only ~30% stallions are considered to freeze well, with the remainder producing varying proportions of live spermatozoa (or none at all) post-thaw. It has been suggested that the high proportion of polyunsaturated acids in sperm membranes makes them susceptible to lipid peroxidation by ROS during the cryopreservation process (Cerolini et al, 2001). An increase in ROS production is believed to be responsible for lipid peroxidation during cryopreservation in ram spermatozoa (Bucak et al, 2007), although Peris et al. (2007) did not find an increase in lipid peroxidation associated with cryopreservation. Therefore, ROS production may depend on breed factors, composition of the semen extender, cooling/warming rates, or a combination of these factors.

Work with human spermatozoa used in fertility treatment has shown that reactive oxygen species (ROS) are present in semen and cause damage to sperm plasma membranes and chromatin (Aitken et al, 1992). The effect of ROS can be reduced by separating viable spermatozoa from cells and cellular debris, and from damaged or dying spermatozoa by density gradient centrifugation (DGC) (Allamaneni et al, 2005). This technique was previously shown to reduce senescence and chromatin damage in human spermatozoa (Morrell et al, 2004). Although DGC is frequently used to prepare human spermatozoa for fertility treatment, it is not often used for animal spermatozoa because of the lack of species-specific formulations and because of the much higher sperm concentrations and/or semen volumes observed with some animal species compared to humans. Recently new colloid formulations for stallion and boar semen have been developed at SLU and, in addition, the centrifugation method for stallion semen has been refined to make it more easily adopted by stud personnel (Morrell et al, 2008). The new method (Single Layer Centrifugation, SLC) selects the most motile, viable and morphologically normal spermatozoa and those with good chromatin integrity (reviewed by Morrell & Rodriguez-Martinez, 2009). Since SLC also separates spermatozoa from SP, it provides an excellent method for studying the effects of SP on spermatozoa. **The aim of this project was to investigate ROS production in stallion sperm samples, both cool-stored and cryopreserved, and to determine potential ways of neutralizing their detrimental effects.**

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Results and Discussion

Funding was granted by the Swedish Foundation for Equine Research (SSH) for two years, starting in January 2008. The work during the 2008 breeding season focused on scaling-up the Single Layer Centrifugation technique (SLC), to enable the removal of sources of ROS to be investigated and freezing experiments to be undertaken. Previously the SLC-technique used 1.5 mL extended equine semen in a centrifuge tube, yielding approximately 50 million motile spermatozoa after SLC-selection. To aid the investigations, and also to increase the attractiveness of the technique for on-stud use, the yield of spermatozoa had to be increased (scale-up), and the ability of the selected spermatozoa to undergo normal capacitation and fertilization required investigation. The preliminary results were presented at the International Symposium for Stallion Reproduction in Brazil in September 2008 and published this year (Morrell et al, 2009, Theriogenology 72, 879-884). A summary of the final results is shown here (Table 1).

Table 1: Effect of scaling-up Single Layer Centrifugation on various parameters of semen quality (n=18)

Parameter	Small-SLC	Inc-SLC	Large-SLC
Motility (%)	88.0 ± 8.8	84.0 ± 3.5	90.0 ± 5.4
Viability (%)	78 ± 11	85 ± 9	81 ± 11.5
DFI (%)	14.7 ± 10.9	12.8 ± 8.1	11.6 ± 7.6
Yield (%)	50 ± 19	53 ± 17	45 ± 18
Normal morphology (%)	69,4 ±12,7	69,4 ±12,9	63,9 ±15,6
Number of tubes needed to process the whole ejaculate	80 x 10 mL	27 x 10 mL	8 x 50 mL

Note: all SLC-preparations were better than the unselected sperm samples (data not shown).

Scale-up continued

During the spring of 2009, aliquots of ejaculates from all the stallions at Flyinge (18 at that time) were used for SLC-Small and SLC-Large. Sperm motility was assessed by computerised sperm motility analysis (CASA) immediately after processing, after 24h and after 48h using the Qualisperm™

Motility Analyser. Total and Progressive motility were better in the SLC-selected samples than in the uncentrifuged controls at all times, as was sperm velocity at 24h and 48h. Therefore, it was possible to use the scaled-up method of SLC successfully on ejaculates from all the stallions tested. These results are currently in press (Morrell et al, Equine Veterinary Journal). A further scale-up to process semen in 100 mL glass tubes was also successful.

Capacitation status of SLC-selected spermatozoa

There have been reports that some sperm processing treatments can induce capacitation, thus shortening the useable life of spermatozoa. Therefore, the effect of SLC on the capacitation status of stallion spermatozoa was examined, using aliquots of the same sperm samples used in the preliminary scale-up experiment. Capacitation status was determined by chlortetracycline-staining and examination of the stained spermatozoa by fluorescence microscopy. SLC-selection did not significantly affect any of the three chlortetracycline staining-patterns; live non capacitated (F), live capacitated (B) or live acrosome-reacted (AR) spermatozoa (Table 2), which is an excellent result. As anticipated, length of storage significantly affected sperm capacitation, in that the proportion of F (non-capacitated) spermatozoa decreased with cold-storage ($P<0.05$) whilst the proportion of B (capacitated spermatozoa) increased ($P<0.05$). The proportion of live acrosome reacted spermatozoa (AR-pattern) also increased significantly with storage ($P<0.05$). The number of non-viable spermatozoa, assessed by ethidium homodimer-1 positive fluorescence, was significantly less in the SLC-selected samples than in the unselected samples at all timepoints (Table 3).

Table 2: CTC-sperm head fluorescence patterns F, B and AR (means ± SD) for cold-storage times of 4, 24 and 72 hours duration.

	4 h		24 h		72 h	
	UN	**SLC**	**UN**	**SLC**	**UN**	**SLC**
F	81±7	85±6	75±8	77±9	64±14	59±13
B	16±6	13±5	23±7	21±8	34±14	38±12
AR	3±2	2±1	2±2	2±2	3±1	3±1

Notes: SLC = Single Layer Centrifugation; F = uncapacitated, acrosome intact;
B= capacitated, acrosome intact; AR= acrosome-reacted. No statistical significance in the banding patterns between the two treatments.

Table 3: Ethidium homodimer-1 positive stallion spermatozoa in the CTC assay (mean ± SD) after cold-storage times of 4, 24 and 72 hours duration.

	4 h		24 h		72 h	
	UN	**SLC**	**UN**	**SLC**	**UN**	**SLC**
EtD-1 positive	22±7	13±5	34±5	15±4	72±4	42±5

The capacitation results were presented as a poster at the ESDAR meeting in September 2009 and a manuscript is in press (Bergqvist et al, Reprod Dom Anim *in press).*

Fertility of SLC-selected spermatozoa

To test the functionality of SLC-selected spermatozoa, they were prepared either by SLC through Androcoll™-E or by density gradient centrifugation (DGC) using a home-made colloid, and were used for intracytoplasmic sperm injection (ICSI) of equine oocytes. This experiment was carried

out at Prof. Cesare Galli´s laboratory in Cremona, Italy, as a collaborative project with this group. The mean cleavage rates of the injected oocytes from SLC and DGC-selected spermatozoa were 66.7% and 66.4% respectively, whilst the proportion of blastocysts developing from cleaved oocytes was 28% and 22% respectively. There was no statistical difference between stallions or between methods. There were slight numerical differences between the two centrifugation methods in their ability to cope with specific morphological defects, for example, spermatozoa with nuclear vacuoles and midpiece defects in the frozen samples, with SLC providing better results than DGC for infertile stallions. These results showed that stallion spermatozoa subjected to SLC-selection on Androcoll™-E have normal functionality and, moreover, SLC may have an advantage over DGC in selecting the normal spermatozoa from ejaculates from sub-fertile stallions. These results are to be presented at the International Symposium on Equine Reproduction in Kentucky, July 2010, and a manuscript has been submitted for publication.

Table **cleavage and development of equine oocytes injected with stallion spermatozoa prepared by either Single Layer Centrifugation or Density Gradient Centrifugation.**

Stallion	Separation methods	Injected	Cleaved	Cleavage rate	Blastocyst d7	Total blastocysts	%Bl/CL	%Bl/Inj
X CTR	DGC	27	17	62,60	2	4	20,60	13,40
X CTR	SLC	28	21	75,00	2	4	19,05	14,29
Y	DGC	20	9	45,00	1	2	22,22	10,00
Y	SLC	20	10	50,00	1	1	10,00	5,00
V	DGC	57	38	66,67	6	6	15,79	10,53
V	SLC	58	34	58,62	7	9	26,47	15,52
W	DGC	47	32	68,09	6	9	28,13	19,15
W	SLC	49	35	71,43	7	12	34,29	24,49

Note DGC = density gradient centrifugation, SLC = single layer centrifugation; Bl = blastocyst; CL = cleavage, Day 7 = 7 days after intracytoplasmic sperm injection.

In further studies to establish the fertility of SLC-selected spermatozoa, the scaled-up SLC method was used to prepare selected sperm doses for artificial insemination (AI), with resulting pregnancies. Ejaculates from five problem stallions were processed by SLC and used for AI, producing a 50% pregnancy rate, compared to 0-20% from previous unselected sperm doses (to be presented at the 2010 ESDAR Conference in Hungary, and a manuscript has been submitted).

Lipid peroxidation in sperm membranes

During the autumn of 2008, stallion sperm samples were prepared using the SLC-Inc technique (4.5 mLs extended ejaculate layered on top of 4 mLs Androcoll™-E), to study lipid peroxidation (LPO) in the sperm membranes using BODIPY staining followed by flow cytometric analysis. Sperm motility was monitored using a Qualisperm™ motility analyser. While sperm motility varied among treatments and stallions and was best in the SLC-selected spermatozoa, the responses to induced LPO, were very low (from 1.09±0.87% to 3.77±0.93%) and variable among stallions, treatments and concentrations of iron sulphate. We concluded that removal of seminal plasma by the SLC procedure

did not significantly increase the susceptibility of stallion spermatozoa to induced LPO in cooled sperm samples. This result is in contrast to the results reported by others for some other species, such as the human and pig, where the presence of ROS is linked to LPO, and in contrast to results with frozen-thawed stallion spermatozoa. Putative explanations for these results are that (i) there are species differences in susceptibility to ROS; (ii) that stallion seminal plasma contains a higher level of antioxidants than non-herbivores, because their diet naturally contains high levels of antioxidants; (iii) the extenders used for stallion semen contain high levels of antioxidants which counteract the effects of ROS. Whatever the explanation, further studies are required to unravel the roles of SP and extender in preventing induction of LPO in equine sperm membranes. These results were presented as a poster at the ESAR meeting in September 2009 and a manuscript has been submitted.

Role of seminal plasma in sperm survival

Experiments were carried out at Flyinge to investigate whether removal of most of the seminal plasma (SP) from a semen sample results in better sperm survival in AI doses. This semen processing technique (sperm washing) is practiced in some countries, e.g. Germany, but is not done routinely in Sweden. The SLC-selected samples had a higher proportion of motile and progressively motile spermatozoa than the uncentrifuged or washed samples at all times (uncentrifuged vs. SLC, $P<0.001$ at all time points for both parameters; total motility, SLC vs. washed, $P<0.01$ at 0h and 48h, $P<0.05$ at 24h; progressive motility, SLC vs. washed, $P<0.01$ at 0h, $P<0.05$ at 24h and $P<0.001$ at 48h). The washed group had better motility than the uncentrifuged samples only at 0h for total motility ($P<0.05$) and only at 48h for progressive motility ($P<0.05$). Sperm velocity was not different between the groups. Total motility and progressive motility fell after storage in all groups but decreased most in the uncentrifuged group. Between 24h and 48h, there was relatively little change in the kinematics of the SLC-selected and sperm washing groups, although all parameters decreased in the uncentrifuged group. The results of the SCSA showed that there was significantly more chromatin damage in washed sperm samples than in the SLC-selected samples. In additional experiments, SP was added to SLC-selected samples. Low proportions (5%) of SP tended to increase progressive motility transiently, although this effect was lost during subsequent storage. Furthermore, there was an increase in chromatin damage where the SLC-selected spermatozoa were stored in the presence of SP. Adding 5% SP to stored SLC-selected spermatozoa increased the beneficial effect on sperm motility, although again the effect tended to be lost with further storage. These results suggest that adding SP to semen doses just prior to AI may increase progressive motility and improve fertility, although the latter needs to be investigated in an AI trial.

The effect of adding various proportions of SP to SLC-selected spermatozoa was also investigated. In a preliminary experiment (n=9), SP was added to SLC-selected spermatozoa in the same proportion as in the uncentrifuged sample (10-50%, depending on the sperm concentration in the original ejaculate). Although mean sperm motility in the SLC+SP group was similar to the SLC-selected sperm group immediately after processing, it was significantly reduced after 24h ($P<0.001$), indicating that 10-50% SP had a negative effect on sperm motility with storage. In a second experiment, lower concentrations of SP (1.5%, 2.5% or 5%) were added to SLC-selected sperm samples. Mean subjective motility was higher in the SLC and SLC+SP groups than in the uncentrifuged group ($P<0.01$ at 0h and $P<0.001$ at 24h) and higher than in the washed group ($P<0.05$ at both times), although there was no significant difference between the washed and the uncentrifuged groups. The SLC and SLC+SP groups were not different. In contrast, mean sperm viability (Table 4) was unchanged in the SLC and SLC+SP groups compared to the uncentrifuged group at both time points. However, sperm viability in the washed treatment group was significantly lower than the other groups ($P<0.01$ at 0h and $P<0.001$ at 24h). There was a correlation between viability and subjective motility for the uncentrifuged group ($P<0.001$) and for the washed group ($P<0.05$) but not for the SLC or SLC+SP groups.

The results of the Qualisperm™ motility analysis were similar to those described for subjective motility, although the degree of significance was higher. There were significant correlations between viability and total motility for the uncentrifuged group ($P<0.001$), the SLC+SP group ($P<0.05$) and the washed group ($P<0.01$) but not for the SLC group. The effect of adding SP at 5%, 2.5% or 1.5% on sperm kinematics was dose-dependent (Table 5), with the addition of 5% SP only having a slight beneficial effect on total motility initially ($P<0.05$), although the benefit was subsequently lost (Figure 2). Sperm viability was not affected by adding SP at these low concentrations, being similar to the SLC treatment group.

Table 4: mean (± SD) subjective motility and viability at two time points for stallion spermatozoa subjected to different processing techniques (n=30).

	Subjective Motility (%)				Viability (%)			
	Uncent	**SLC**	**SLC+SP**	**wash**	**Uncent**	**SLC**	**SLC+SP**	**wash**
0h	70±10 [a]	79±12 [a]	75±14 [b]	68±11 [ab]	74.7±10	74.3±10	75.9±10	66.4±12 [a]
24h	56±12 [c]	70±12 [c]	68±13 [bc]	59±13 [b]	66.5±10	69.2±8	69.7±9	58.8±12 [b]

Note: uncent = uncentrifuged sperm samples, SLC = Single Layer Centrifugation; SLC+SP = seminal plasma added to the selected spermatozoa after Single Layer Centrifugation to give the same ratio as in the uncentrifuged sample; washing = centrifugation without a colloid; a = significantly different from all other groups ($P<0.01$), b = significantly different from all other groups ($P<0.001$).

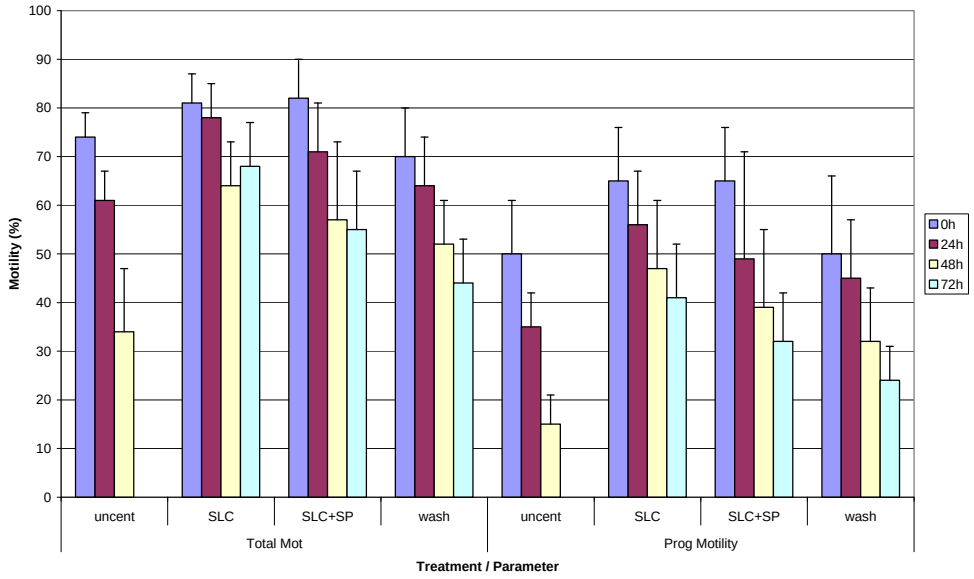
Table 5: Effect of adding different proportions of seminal plasma to SLC-selected spermatozoa on % total and progressive motility (n=30)

	Un	SLC	SLC +5%SP	Un	SLC	SLC +2.5%SP	Un	SLC	SLC +1.5%SP
TM 0h	72±9	77±11	**82±9**	73±9	85±4	83±6	74±5	81±6	82±8
TM 24h	63±15	70±13	65±16	56±10	71±16	75±10	61±6	78±7	71±10
PM 0h	49±10	56±19	65±15	54±14	61±14	55±21	50±11	65±11	65±11
PM 24h	35±15	53±19	50±16	39±14	53±17	55±15	35±7	56±11	49±22

Note: Uncent = uncentrifuged, SLC = Single Layer Centrifugation, SLC+%SP = = Single Layer Centrifugation with either 1.5%, 2.5% or 5% seminal plasma added to the sperm pellet. TM = total motility (%), PM = progressive motility (%). Figure in bold indicates statistical significance $P<0.05$ (i.e. only for 5% SP at 0h).

From these results it can be seen that selecting the most robust spermatozoa by SLC gives better results in terms of motility and sperm membrane integrity than centrifuging spermatozoa to remove SP (sperm washing). Furthermore, there was no evidence to suggest that maintenance of sperm motility and viability could be enhanced by adding back SP to SLC-selected spermatozoa. On the contrary, longer-term cold storage of spermatozoa in the presence of small concentrations of SP resulted in a reduction in total motility and progressive motility compared to SLC alone. High concentrations of SP were detrimental to sperm survival, even for SLC-selected spermatozoa. These results are currently in press (Morrell et al, Reproductive Biomedicine Online).

Figure 2: Effect of Single Layer Centrifugation, seminal plasma (1.5%) and sperm washing on stallion mean values (±SD) of sperm kinematics (n=10)



Note: uncent = uncentrifuged sperm samples, SLC = Single Layer Centrifugation; washing = centrifugation without a colloid; SLC+SP = seminal plasma added to the selected spermatozoa after Single Layer Centrifugation to give approximately the same ratio as in the washed sample (1.5%).

Reactive Oxygen Species in stallion semen

In collaboration with a group in Spain, it was shown that ROS do not cause lipid peroxidation in stallion spermatozoa, in contrast to previous findings in boar spermatozoa. These results may be due to the diet being high in natural anti-oxidants, or to the use of high levels of antioxidants in semen extenders (C. Ortega Ferrusola et al, submitted).

Although it was not possible to establish the luminometric assay for reactive oxygen species (ROS) at SLU, as reported in 2008, a flow cytometric assay using the fluorescent dyes hydroethidine and 2′,7′-dichlorodihydrofluorescein (Guthrie & Welch, 2006) has now been established at SLU. In a preliminary study, the relationship between ROS in seminal plasma and lipid peroxidation in sperm membranes was studied using frozen-thawed semen from two stallions (both considered to be poor freezers), obtained from Flyinge AB. The spermatozoa from both stallions had poor viability on thawing, and showed ROS-production among cells considered viable, which increased with time after thawing (Table 6). However, the latter did not induce significant lipid peroxidation, in keeping with our previous results with cooled spermatozoa.

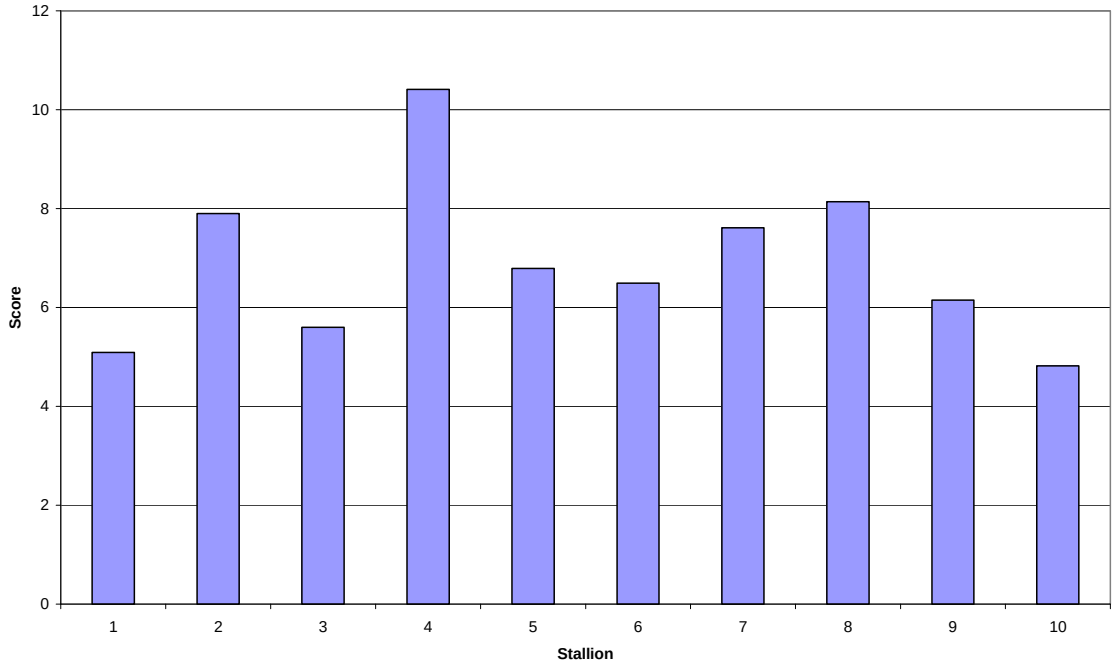
Table 6: Viability, ROS-production and lipid peroxidation in two stallions

	% alive (0h)	% alive (2h)	% ROS (0h)	% ROS (2h)	% lipid perox (0h)	% lipid perox (2h)
Stallion 1	18	11	**51**	84	1.3	0.7
Stallion 2	24	13	49	83	1.5	1.9

The ROS assay, in conjunction with SYBR-14/propidum iodide staining for sperm viability and CASA, was subsequently used to evaluate the post-thaw quality of cryopreserved semen from 10

stallions, two of which were of known poor fertility. In the frozen-thawed samples, viability was correlated with the proportion of ROS-negative, viable spermatozoa i.e. spermatozoa that were not producing ROS ($P<0.05$), and viability was also correlated with progressive motility ($P<0.01$). When a scoring system was used to create an index based on several parameters of sperm quality, including ROS-negativity, the stallions with poor fertility had lower scores than most of the others (Figure 3).

Figure 3: Index of different parameters of sperm quality used to assess frozen-thawed stallion semen.



Note: stallions 3 and 10 are of known poor fertility following cryopreservation, the others have not been tested yet in AI.

Recently, new commercial cryoextenders have become available containing amides instead of glycerol as cryoprotectant. It is considered that these amides should cause less osmotic shock to spermatozoa and therefore, less ROS production. In collaboration with a group in Belgium, split ejaculates were used to prepare cushion-centrifuged and SLC-selected samples for cryopreservation. On thawing, the SLC-selected samples had higher total motility and higher progressive motility than the cushion-centrifuged samples (total motility 46.5% vs. 29.7%; progressive motility 31.9% vs.18.6%; M. Hoogewijs, unpublished data). Strict assessment criteria were used, with ≥30% progressive motility being considered acceptable for AI. On an individual basis, all SLC-selected samples had higher progressive motility than their cushion-centrifuged counterparts. These results are very encouraging for the development of new, freezing protocols which are effective for ejaculates that have not been frozen successfully yet. Further work is necessary to optimize the freezing protocols for SLC-selected spermatozoa and to test their fertility after AI.

In conclusion, the project has produced interesting results of practical importance to equine breeding, and enabled several new collaborations, both national and international, to be established. The results have also raised further questions of importance to equine AI, such as the importance of seminal plasma to mare fertility, which need to be resolved in further studies.

Papers arising from the project (published, or accepted for publication)

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