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A simplified fixed-time insemination protocol using frozen–thawed stallion spermatozoa stored at 17°C for up to 24 h before insemination

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Abstract

Background: Insemination of mares with frozen–thawed spermatozoa requires intensive management and results in 40%–60% per cycle pregnancy rates.

Objectives: To determine if satisfactory fertility is possible for frozen–thawed semen after processing it through a microfluidic device, followed by storage at 17°C for up to 24 h before fixed-time insemination.

Study design: Uncontrolled field trials.

Methods: A pilot study evaluated the motility of frozen–thawed spermatozoa after centrifugation and storage (17°C) in two different media for up to 48 h. Subsequently, the motility of frozen–thawed semen processed through a microfluidic device, resuspended in two different media during storage (17°C) for up to 24 h was evaluated. The fertility of frozen–thawed spermatozoa, after microfluidic sorting and storage at 17°C for up to 24 h, was evaluated after fixed-time insemination in a commercial embryo programme. Experiment 1: Frozen–thawed spermatozoa ($N = 5$ stallions) were centrifuged and resuspended in Botusemen Gold™ or SpermSafe™ and stored (17°C) for up to 48 h. Sperm motility was evaluated by CASA at 0, 6, 24 and 48 h. Experiment 2: Frozen–thawed spermatozoa ($N = 4$ stallions) underwent microfluidic sorting and storage (17°C) for up to 24 h in both media. Sperm concentration and motility were evaluated at 0, 16 and 24 h. Experiment 3: Fertility of frozen–thawed spermatozoa ($N = 3$ stallions) was evaluated after insemination of 42 mare cycles at 6, 16 and 24 h after thawing, microfluidic sorting and storage before fixed-time insemination.

Results: The stallion significantly influenced sperm motility, but there was no effect of media on motility parameters. Storage time significantly affected sperm motility after centrifugation but not after microfluidic sorting. Storage time had no effect on the overall embryo recovery rate (52%, $n = 42$).

Main limitations: Field trial with small mare numbers and no control at time = 0 h.

Conclusions: Fixed-time insemination of frozen–thawed spermatozoa after microfluidic sorting and storage at 17°C for up to 24 h produced satisfactory embryo recovery rates.

KEYWORDS
fixed-time insemination, frozen semen, horse, microfluidic sorting

1 | INTRODUCTION

Frozen semen has become increasingly popular in sport horse breeding programmes and per cycle conception rates range from 40% to 60% when used in commercial breeding programmes.[1,2] There are three major issues with frozen stallion spermatozoa that concern breeders: (1) The fertility of frozen stallion sperm is lower than that of fresh spermatozoa due to its poor longevity after thawing; (2) To compensate for this reduced sperm longevity, intensive and expensive veterinary management of the mares is required to optimise fertility; and (3) The logistics and high costs associated with the transport of frozen spermatozoa in expensive and potentially hazardous liquid nitrogen shippers. The cost of shipping a small recyclable box containing a thawed dose of semen overnight is 5%–10% of the cost of shipping dry vapour shippers to the breeding farm and back to the semen storage centre. With the development of new semen longevity extenders, opportunities to develop practical and better economic solutions for the management and transport of frozen–thawed stallion spermatozoa are worth investigating.

Typically, the insemination of mares with frozen spermatozoa involves intensive reproductive management with multiple examinations of follicle development around the time of ovulation.[1] It is recommended that a commercial insemination dose of frozen spermatozoa should contain a minimum of 250 million progressively motile spermatozoa and exhibit progressive motility above 35%.[3] It has been reported that single insemination of a total of 800 million frozen–thawed spermatozoa within 12 h of ovulation can produce per cycle pregnancy rates of 44.7% (55/123).[4] Other studies have performed fixed-time insemination with commercial doses of frozen–thawed semen, providing a minimum of 200 million motile spermatozoa at 32 h after human chorionic gonadotropin (hCG) administration or at 40 h after gonadotropin-releasing hormone (GnRH) analogue and achieved pregnancy rates of 54.7%[5] and embryo recovery rates of 40%.[6] The use of a split fixed-time insemination protocol at 24 and 40 h after ovulation induction, with a total of 800 million frozen–thawed spermatozoa, has been shown to be useful for stallions with both good and poor fertility.[7] Previously, it has been shown that when lower doses of frozen–thawed spermatozoa are used, high pregnancy rates are achievable when mares are inseminated either conventionally (67%) or hysteroscopically (64%) with low doses containing 14 million frozen–thawed spermatozoa at a fixed time of 32 h after hCG administration.[8] In this same study, when the insemination dose was reduced to 3 million frozen–thawed spermatozoa, hysteroscopic insemination produced better results (47%) than conventional insemination (15%).

More recently, it has been reported that post thaw motility >30% can be maintained at 5°C for 24 h and pregnancies[9] (9/16 cycles, 56%) have been achieved after thawing, centrifuging and storing frozen–thawed stallion semen at 5°C for up to 24 h.[9,10] In addition, new methods for the liquid storage of fresh stallion spermatozoa at room temperature in protein-free media[11] may provide opportunities for improving the longevity of frozen–thawed spermatozoa.

Cryopreservation of stallion semen is associated with significant cell damage due to the effects of freeze–thaw injury[12] and reactive oxygen species.[13] Therefore, to optimise the longevity of frozen–thawed spermatozoa, it is desirable to select a high proportion of viable spermatozoa. New microfluidic sperm sorting methods used in in vitro embryo production systems have been shown to select subpopulations of frozen–thawed spermatozoa with high motility,[14] normal morphology, viability, membrane integrity and DNA integrity without the need for centrifugation as required for other sperm separation techniques like density gradient separation.[15,16] Centrifugation of spermatozoa has been shown to increase the level of reactive oxygen species in the sample, which is detrimental to sperm DNA integrity and fertility[17] and so the ability to eliminate a centrifugation step may be beneficial. Microfluidic sperm separation, therefore, offers a very practical and economic method of selecting a viable population of spermatozoa without the need for centrifugation.

The optimal temperature for storing stallion semen has not been fully determined, but various reports suggest it ranges from 5°C to 20°C.[11,18] For fresh semen, it is recommended that the cooling rate during the phase transition of the sperm membrane is −0.5°C/min, and faster cooling rates may induce cold shock, which is detrimental to sperm viability. The mean temperature during December in New Zealand ranges between 20°C and 25°C (niwa.co.nz), and overnight the temperature ranges from 10°C to 17°C, therefore providing an ambient temperature within the range suitable for sperm storage. During the freezing and thawing process, spermatozoa experience oxidative damage and so a storage temperature of 17°C after thawing may also minimise potential membrane damage due to peroxidation, excess calcium influx and membrane phospholipid disorder, which occurs at high or low temperatures.[19] The protein-free sperm longevity media, SpermSafe™, was designed to maintain fresh sperm viability at this temperature (17°C), but there are no reports of its ability to maintain the viability of frozen–thawed spermatozoa over time.

The objective of this study, therefore, was to determine if satisfactory fertility was achievable in a commercial embryo programme by combining microfluidic sperm isolation techniques with the protein-free ambient temperature sperm longevity media and a simple fixed-time insemination protocol, to reduce the costs of shipping frozen semen and the intensive veterinary management of mares for frozen semen insemination.

2 | MATERIALS AND METHODS

2.1 | Semen preparation

2.1.1 | In vitro semen evaluation

Experiment 1: Three straws (0.5 mL) of frozen semen from stallions of proven fertility ($N = 5$, $r = 2$) were thawed at 37°C for 30 s in a water bath and diluted in 1:1 (vol/vol) of Botusemen Gold™ (Botupharma Ltd.). The thawed semen was centrifuged at 400 g for 4 min at room temperature and the 100 µL pellet of spermatozoa was resuspended in either 1 mL of SpermSafe™ (Breed Diagnostics) or Botusemen Gold™. This sample was stored at 17°C and evaluated for motility and

concentration by Androvision™ (Minitube) computer-assisted semen analysis software at 0, 6, 24 or 48 h after thawing. Semen was stored at 17°C in a portable car fridge (Ridge Ryder Thermo Cooler/Warmer 12 L, Supercheap Auto Pty Ltd).

Experiment 2: Three straws (0.5 mL) of frozen semen from stallions of proven fertility ($N = 4$, $r = 2$) were thawed at 37°C for 30 s in a water bath and diluted in 1:1 (vol/vol) of Botusemen Gold™. The thawed semen was expelled into a test tube and 2 mL of warm (37°C) Botusemen Gold™ was added to the 3 mL of thawed sperm suspension. An aliquot of 1 mL of thawed semen diluted in Botusemen Gold™ was reserved as an untreated control. An aliquot of 3 mL of thawed diluted spermatozoa was aspirated into a 5 mL syringe and spermatozoa were infused into the microfluidic device (VetMotl™, Inc.) via the inlet port.

The outlet port was primed with 100 µL of either Botusemen Gold™ or SpermSafe™ semen diluent and 2.9 mL of the respective semen diluent was added dropwise onto the surface of the membrane from a height of 2 cm so that the media covered the membrane. The device was placed in a humidified petri dish and incubated in the dark at room temperature for 30 min. A 1 mL syringe was attached to the outlet port and a total volume of 1 mL was aspirated into the syringe. The 1 mL sample of diluted spermatozoa was stored in a flat bottom 5 mL polypropylene vial at 17°C until the time of evaluation. After thawing, sperm concentration and motility parameters were evaluated before and after processing through the microfluidic device and at 0, 16 and 24 h after thawing and compared with an untreated control sample diluted in Botusemen Gold™ but not processed through the microfluidic device.

Experiment 3: Semen preparation for insemination.

Three straws (0.5 mL) of frozen semen from three stallions from the same stud with proven fertility were thawed and diluted in 1:1 (vol/vol) Botusemen Gold™. The semen was processed through the microfluidic device and stored in SpermSafe™ diluent at 17°C for up to 24 h. The sperm concentration of the samples and the motility parameters were evaluated by Androvision™ (Minitube) computer-assisted semen analysis at 0, 6, 16 and 24 h after thawing.

Insemination study

The frozen–thawed spermatozoa were prepared as described above. The 1 mL sample of spermatozoa diluted in modified, protein-free SpermSafe™ medium was stored in a flat bottom 5 mL polypropylene vial at 17°C until the time of insemination. After thawing, processing and storing, spermatozoa from three commercial stallions (one batch per stallion) were used to inseminate a total of 42 mare cycles following sperm storage for 6 ($N = 13$), 16 ($N = 16$) or 24 ($N = 13$) hours after thawing and processing.

2.1.2 | Mare management and insemination

This study was undertaken in the Waikato region of New Zealand (37.78° S, 175.25° E). All mares in this breeding programme were fed ad lib baylage and 2 kg pellets (NRM Evolve™ nuts; NRM) daily.

For this study, non-lactating, reproductively normal mares ($N = 11$ mares, 42 cycles) ranging from 5 to 12 years old were selected based on their history of conceiving and foaling uneventfully in the last 2 years. The mares' reproductive tracts were examined daily by per rectal ultrasound during oestrus until a dominant 35 mm follicle, uterine oedema and a soft cervix were detected. At this time, they were treated with 1.25 mg Deslorelin IM (BioRelease® Deslorelin; Dechra Ltd). The mares' reproductive tracts were examined by transrectal ultrasound 22 h after ovulation induction treatment to confirm suitability for insemination, again at 40 h, at the time of insemination and finally 24 h after insemination to confirm ovulation and to determine if any intrauterine fluid was evident (Figure 1). At 24 h after insemination the mares were treated once with 10 IU oxytocin subcutaneously.

At 40 h after ovulation induction, the mares were inseminated using a deep horn insemination technique to deliver the 1 mL insemination dose—which contained a mean (±SEM) of 9.26 (±1.62) $\times$ 10^6 total spermatozoa in 2 $\times$ 0.5 mL straws, ipsilateral to the side of the dominant follicle or ovulation. The deep uterine insemination technique involved passing a flexible equine universal pipette (Catalogue #17209/0001; Minitube) through the cervix and manipulating it per rectum along the lumen of the uterine horn ipsilateral to the dominant follicle and depositing the insemination dose (1 mL) at the tip of the uterine horn.[20]

To evaluate the fertility of the frozen–thawed, stored spermatozoa, embryos were recovered from the uterus by transcervical uterine lavage.[21,22] This was performed using a cuffed silicone catheter (French size 33) passed through the cervix into the uterine lumen. The uterus was flushed with 3 $\times$ 1 L sterile compound sodium lactate (Baxter™; SVS Veterinary Supplies) and the embryo was collected in a Miniflush® filter system (mesh size 40 µm; Catalogue #19222/2000; Minitube) at 8 days after ovulation.

During the same breeding season, the reproductive tracts of 37 commercially available mares (5–18 years old) were monitored by ultrasound every 6 h after ovulation induction with 1.25 mg Deslorelin IM (BioRelease® Deslorelin; Dechra Ltd). These mares were inseminated when ovulation was confirmed with a minimum of 200 million total motile frozen–thawed spermatozoa from stallions ($N = 15$) from the same stud.

2.1.3 | Data analysis

Analysis of the sperm motility data was conducted using the Minitab v20 general linear model analysis model to identify treatment effects. To evaluate the effects of before and after treatment on the same sample a paired *t*-test was also used. The normal distribution of residuals was assessed using a Ryan-Joiner test. In cases where residuals deviated from normal distribution and no interactions between factors were observed, a Johnson transformation was used. Differences between means were considered significant when $p \leq 0.05$. Comparisons of means were performed using the Tukey comparison method.

In Experiment 1, the sperm motility parameters were evaluated post thawing at $t = 0$ h and then at $t = 6$, 24, 48 h after processing

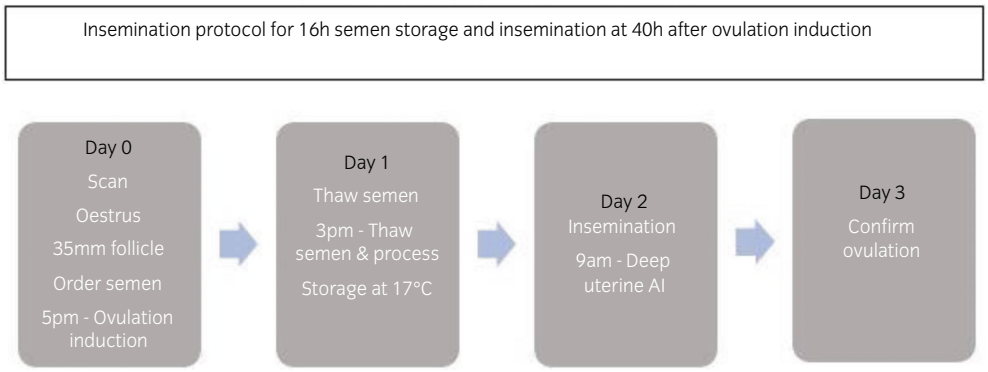

FIGURE 1 Insemination protocol.

TABLE 1 Experiment 1: CASA motility parameters following the centrifugation and storage of frozen–thawed stallion spermatozoa ($N = 5$ stallions, $r = 2$) in either Botusemen Gold or SpermSafe at 17°C at 0, 6, 24 and 48 h.

Post thaw storage time	Motility parameter (%)	Botusemen Gold™	SpermSafe™
0 h	Total motility	22.1 ± 4.9	24.9 ± 5.2
	Progressive motility	15.9 ± 3.5	18.3 ± 4.3
	Rapid motility	3.3 ± 0.7	6.1 ± 1.6
6 h	Total motility	21.4 ± 3.4	22.2 ± 4.8
	Progressive motility	17.9 ± 2.9	17.0 ± 4.0
	Rapid motility	7.1 ± 1.1	5.8 ± 1.3
24 h	Total motility	19.3 ± 4.0	17.8 ± 3.8
	Progressive motility	14.5 ± 3.2	13.2 ± 2.7
	Rapid motility	4.4 ± 1.1	5.2 ± 1.2
48 h	Total motility	11.9 ± 2.4	12.8 ± 3.6
	Progressive motility	9.5 ± 2.2	9.4 ± 3.0
	Rapid motility	2.4 ± 0.6	3.7 ± 1.2

Note: No significant differences were observed between media at any time point for any motility parameter.

and storage at 17°C. In Experiment 2, the sperm motility parameters and concentration were evaluated post thawing at $t = 0$ h and then again at $t = 16$ and 24 h after processing through the microfluidic device and storage at 17°C and compared to untreated control. For Experiment 3, the insemination study, the sperm motility was evaluated immediately after thawing and before processing through the microfluidic device, then after processing at $t = 6$, 12, 24 h. The sperm concentration was evaluated using computer-assisted semen analysis (Androvision software; Minitube) before and after processing through the microfluidic device. The effect of processing spermatozoa through the microfluidic device on in vitro sperm motility (computer-assisted sperm assessment; AndroVision®; Minitube) before and after processing was analysed by a paired *t*-test (results presented as mean ± SEM). The differences in embryo recovery rates by stallion, relative to ovulation and at each semen storage time point 6, 16 and 24 h, were evaluated by chi-square analysis.

3 | RESULTS

Experiment 1: After thawing, centrifuging and storing the spermatozoa in either Botusemen Gold™ or Spermsafe™ medium, significant effects of stallion and storage time were revealed for total motility ($p \leq 0.01$ and 0.05, respectively), progressive motility ($p \leq 0.001$ and 0.05, respectively) and rapid motility ($p \leq 0.01$ and 0.01, respectively), while significant effects of storage time only were revealed for local motility ($p \leq 0.05$) (Table 1). No effect of the medium was revealed, and there was also no evidence of interactions between any of the following factors: stallion, post thaw storage time or medium.

Experiment 2: After thawing, diluting semen in Botusemen Gold™ medium, processing through the microfluidic device and resuspending the spermatozoa in either Botusemen Gold™ or SpermSafe™ medium and compared to a non-processed control sample, there was a significant effect of stallion on the total motility ($p < 0.001$) and progressive motility ($p < 0.001$) (Table 2). No effect of the medium was revealed, and there was also no evidence of interactions between any of the following factors: stallion, post thaw storage time or medium.

Experiment 3: There was a significant beneficial effect of processing the thawed spermatozoa through the microfluidic device on total sperm motility (65.1 ± 9.8% vs. 76.1 ± 10.52% for pre- and post-processed spermatozoa, respectively; $p < 0.001$), progressive motility (pre-processed: 55.5 ± 11.7% and post-processed: 72.4 ± 10.4%; $p < 0.001$; Table 3), rapid motility

TABLE 2 Experiment 2: CASA motility parameters following processing thawed semen through microfluidic device and storage of frozen–thawed stallion spermatozoa ($N = 4$ stallions, $r = 2$) in either Botusemen Gold or SpermSafe at 17°C at 0, 16 and 24 h.

Post thaw storage time	Motility parameter (%)	Control	Botusemen Gold after microfluidic sperm selection	SpermSafe after microfluidic sperm selection
0 h	Total motility	50.0 ± 15.1	55.0 ± 9.6	60.0 ± 4.1
	Progressive motility	43.1 ± 13.4	44.3 ± 10.0	42.0 ± 5.4
16 h	Total motility	53.1 ± 13.2	51.3 ± 18.9	41.5 ± 13.9
	Progressive motility	41.4 ± 11.9	44.7 ± 16.9	39.6 ± 13.2
24 h	Total motility	43.5 ± 14.0	57.1 ± 3.7	60.8 ± 11.7
	Progressive motility	35.7 ± 12.8	56.17 ± 3.0	59.7 ± 11.0

Note: Data presented as mean ± SEM. No significant differences were observed between media at any time point for any motility parameter.

TABLE 3 Experiment 3: Effect of processing frozen–thawed spermatozoa ($N = 3$ stallions, $r = 2$) through the microfluidic device.

Semen parameter	Before microfluidic sperm selection	After microfluidic sperm selection	*p* Value
Total motility %	65.1 ± 2.1	76.1 ± 2.2	<0.001
Progressive motility %	55.5 ± 2.4	72.4 ± 2.2	<0.001
Rapid motility %	13.1 ± 1.5	48.0 ± 2.8	<0.001
Sperm concentration ($\times 10^6$/mL)	114.8 ± 9.2	9.3 ± 1.6	<0.001

Note: Data presented as mean ± SEM. Paired *t*-test, the Johnson transformation was performed on non-normal data, significance is denoted when $p < 0.05$.

(pre-processed: 13.4 ± 7.4% and post-processed: 48.0 ± 13.5%; $p < 0.001$). There was a significant effect ($p \leq 0.001$) of stallion and storage time (0, 6, 16, 24 h) after thawing on the sperm total, progressive and rapid motility. Both the total and progressive motility parameters were maintained above 55% for 24 h post thaw (Figures 2 and 3).

There was a significant effect of microfluidic processing on sperm concentration, with a mean pre-processing sperm concentration of 114.8 ± 44.2 × 10^6 spermatozoa/mL and a mean post-processing sperm concentration of 9.3 ± 7.8 × 10^6 spermatozoa/mL ($p \leq 0.001$). This translates to a 1 mL insemination dose containing a mean of 9.3 × 10^6 spermatozoa. There was no effect of stallion on the sperm concentration before or after processing through the microfluidic device.

3.1 | Insemination and embryo recovery study

The overall embryo recovery rate was 52% ($N = 42$ mare cycles). The embryo recovery rates ranged from 31% to 69% for the three storage time treatment groups, but these differences were not statistically significant (Table 4), nor were they different between stallions (Table 5). Only one mare developed post-breeding accumulation of uterine fluid as diagnosed by ultrasound and she was excluded from the trial. None of the other mare cycles ($N = 42$) exhibited any evidence of or required any treatment for, intrauterine fluid accumulation after insemination.

There was no effect of the timing of insemination (with respect to the time of ovulation) on the per cycle embryo recovery rate. At the time of insemination (40 h post ovulation induction treatment), 17/42 (40%) mare cycles had ovulated and 25/42 (59.5%) mare cycles had not yet ovulated. There was no difference in the embryo recovery rates from mares that had (52.9% embryos/cycle) or had not ovulated (52.0% embryos/cycle) at the time of insemination. None of the mares produced more than one embryo per recovery. There was no effect of mare, cycle number or date of insemination on the embryo recovery rate.

The per cycle conception rate of a reference group of commercially bred mares ($N = 37$) inseminated within 6 h post ovulation using 200 million frozen–thawed spermatozoa from stallions at the same stud was 52.6% ($N = 57$ cycles). In the commercial embryo breeding programme, 8 out of 13 mares produced embryos (61.5%) after deep uterine insemination within 6 h post ovulation with a minimum of 200 million motile frozen–thawed semen from the same three stallions (A: $N = 1$, B: $N = 3$, C: $N = 9$).

4 | DISCUSSION

Our study demonstrates that a fixed-time insemination protocol including a microfluidic selection of frozen–thawed spermatozoa followed by storage of frozen–thawed stallion spermatozoa in modified SpermSafe™ diluent at 17°C for 6–16 h can produce satisfactory embryo recovery rates after low dose, deep uterine insemination at 40 h after ovulation induction. The overall embryo recovery rates (52%) achieved compared favourably with the per cycle pregnancy rates achieved by both our reference group of mares, and those reported after insemination of frozen semen at a fixed time before ovulation (54.7%) or within 6 h post ovulation (38.3%) under New Zealand conditions.[5] Both studies, however, were field studies without a control group. In another study which evaluated the pregnancy rates achieved using an intensive monitoring post ovulation insemination protocol versus double insemination at fixed times of 24 and 40 h after treatment with deslorelin, the overall pregnancy rates were 41% for post ovulation insemination and 52% for double fixed-time insemination.[7]

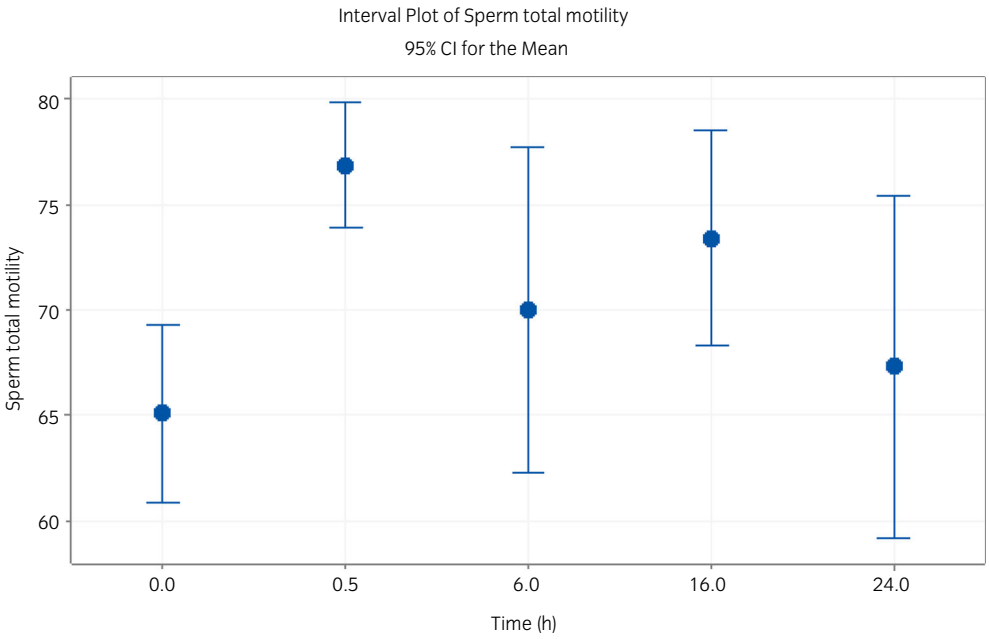

FIGURE 2 Experiment 3 insemination trial: % sperm total motility over 24 h post thaw ($N = 3$ stallions, 42 cycles).

Individual standard deviations are used to calculate the intervals.

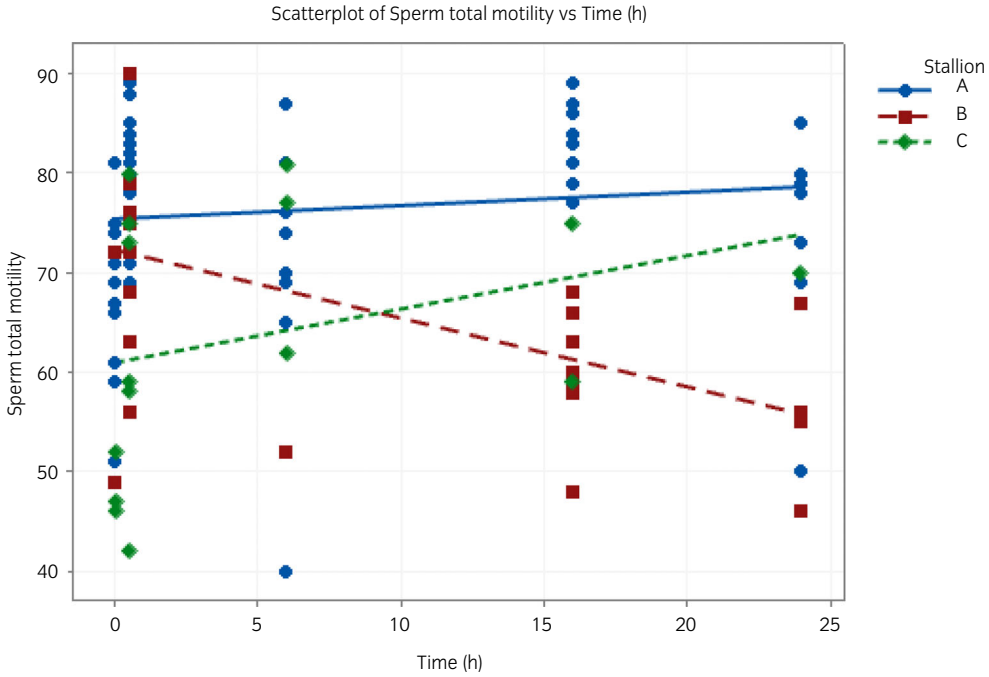

FIGURE 3 Experiment 3 insemination trial: % total sperm motility over time for each stallion ($N = 3$) over time.

TABLE 4 Embryo recovery rates (%) for each treatment group (hours after thawing).

Duration of spermatozoa storage after thawing (h)	Mares inseminated (*N*)	Embryos recovered (*N*)	Embryo recovery (%)
6	13	7	53.8%
16	16	11	68.8%
24	13	4	31.0%
Total	**42**	**22**	**52.4%**

In an effort to reduce the frequency of veterinary monitoring of follicle development and ovulation, the number of examinations was reduced from six hourly in the reference group of commercial mares to once daily for the mares in this trial. Consequently, the 18 h interval between the ultrasound examination of the reproductive tract at 22 and 40 h after ovulation induction was strategically intended to fit in with the courier schedule. Nevertheless, despite finding that 40% of the mares had ovulated at the fixed time of insemination, we observed no difference in the embryo recovery rates of mares that had ovulated before or after insemination. The insemination time of

TABLE 5 Embryo recovery rates (%) for each stallion.

Stallion	Total mares inseminated (*N*)	Embryos recovered (*N*)	Embryo recovery (%)
A	24	14	58.3%
B	13	5	38.5%
C	5	3	60.0%
Total	**42**	**22**	**52.4%**

40 h after ovulation induction with the GnRH agonist was selected based on its expected proximity to ovulation, as it has been reported that the mean time to ovulation from deslorelin treatment was 38.6 h[23] to 40.9 h[7] in oestrous mares with a >30 mm follicle at the time of treatment.

Due to the limited group sizes in this breeding programme, there was no significant difference in the embryo recovery rates across the three time periods. Therefore, further investigation of the fertility of the thawed stored spermatozoa for 24 h or longer is warranted. Our overall sperm motility results are like those obtained by others using frozen–thawed semen stored at 5°C for 24 h.[9,10] Together, these studies pave the way for a new approach to inseminating mares with frozen–thawed sperm and it appears to be very feasible to reduce the intensity of the veterinary management of mares inseminated with frozen–thawed semen.

The mare management and fixed-time insemination protocol used in this study was very simple, requiring only one ultrasound examination of the reproductive tract on the day of insemination. Furthermore, the low incidence of intrauterine fluid accumulation observed after insemination, despite the insemination of the mares on more than one oestrous cycle with frozen–thawed semen, was a very positive outcome. Previous reports have suggested that the removal of seminal plasma from the semen freezing and thawing process reduces the immunomodulation effects of seminal plasma and increases the risk of persistent breeding-induced endometritis.[6,24,25] Therefore, when mares were examined at 24 h after insemination, there was no ultrasonographic evidence of intrauterine fluid accumulation, and therefore, no indication for treatment with uterine lavage or intrauterine antibiotics, which are commonly used in veterinary stud practice. This may be due to the fact that the peak physiological and transient post-breeding inflammation induced by spermatozoa occurs at around 6 h post insemination[26] and it had resolved by 24 h in this reproductively normal group of mares.

There was also no effect of cycle number on the embryo recovery rates from this group of mares as their fertility was maintained. This beneficial outcome may be related to the use of fertile mares resistant to persistent breeding-induced endometritis, the use of the protein-free SpermSafe™ media accompanied by the low number and concentration of spermatozoa used for deep insemination. Foreign protein stimulation of the uterus, sperm number and insemination method have been previously reported to influence the incidence of persistent breeding-induced endometritis.[4,27,28]

In the present study, processing frozen–thawed stallion spermatozoa using the microfluidic device enabled a very small population of viable spermatozoa to be stored at 17°C for up to 24 h. This new microfluidic device has previously been evaluated for in vitro embryo production programmes for the selection of viable spermatozoa before intra-cytoplasmic sperm injection (ICSI).[29] While ICSI requires very low sperm numbers, artificial insemination requires much higher numbers to achieve acceptable fertility rates. Nonetheless, despite recovering only approximately 10% of the total sperm population available after thawing, in this study, the highly selected small population of spermatozoa produced satisfactory fertilisation in vivo, reflected by commercially acceptable embryo recovery rates. It has previously been shown that artificial insemination with low numbers of spermatozoa ($<14 \times 10^6$) can produce satisfactory pregnancy rates using frozen–thawed spermatozoa that have been processed through a Percoll density gradient followed by hysteroscopic insemination at a fixed time of 32 h after ovulation induction.[8] With improvements in deep uterine insemination techniques since 2003, hysteroscopic insemination is no longer required, making the protocol described in this study very practical to implement in commercial practice. Moreover, the simple protocol for processing the frozen–thawed spermatozoa with the microfluidic device did not require a centrifugation step and effectively selected a viable population of spermatozoa.

Our preliminary investigation of the sperm motility parameters throughout 24–48 h storage after thawing revealed that simply centrifuging the thawed sample and resuspending it after thawing will not provide satisfactory motility (>35%) for all stallions when stored at 17°C. In this study, we observed that stallion differences and storage time influenced sperm motility after thawing, but there was no effect of storage media on sperm motility. Other studies have shown that centrifugation of thawed semen followed by storage at 5°C is capable of maintaining the post thaw motility above 30% for up to 24 h.[9] In Experiment 2, we observed that the sperm motility was maintained above 35% similarly for both media for 24 h after processing the thawed semen through the microfluidic device. Despite the selective process provided by the microfluidic device, stallion differences in motility were not eliminated. Again, in the insemination trial using another group of stallions, the effect of stallion and storage time on the sperm motility parameters was evident after processing thawed semen through the microfluidic device and storing it in the protein-free SpermSafe™ medium.

Based on the understanding of the effects of oxidative stress on the longevity of stallion spermatozoa, this protein-free sperm storage medium can optimise the fertility and longevity of fresh spermatozoa from stallions for up to 14 days[30,31] and creates opportunities to improve the longevity and fertility of frozen–thawed, sex-sorted or sub-fertile spermatozoa. In a recent report,[32] 67% of mares conceived after insemination with spermatozoa from a stallion with poor cooling tolerance, which was processed and extended with SpermSafe™ in comparison with no pregnancies achieved with another commercially available milk-based stallion semen extender. Given that there was no significant difference in the sperm motility parameters between post thaw samples processed using a casein-based media or the protein-free media and having limited numbers of mares available for the trial, the protein-free media designed for sperm longevity was selected for fertility evaluation.

The combination of sperm isolation using the microfluidic device, and post-isolation storage in the protein-free modified SpermSafe™ medium simplifies the logistics and reduces the expense associated with transporting frozen spermatozoa. Based on the effect of each stallion observed for the sperm motility parameters over time, it would be recommended to assess the suitability of different stallions for this protocol before commercial application. The number of 0.5 mL straws provided per insemination dose under commercial conditions typically ranges from 1 to 8 straws to provide a minimum of 200 million motile spermatozoa in a dose. For this study, 3 × 0.5 mL straws of commercially frozen spermatozoa were processed to create one insemination dose, which contained approximately 9 million spermatozoa (1 mL). It may be possible to compensate for variability in stallion fertility by increasing the sperm numbers or number of straws available for processing and deep uterine insemination.

In conclusion, the ability to thaw frozen spermatozoa and store it at an ambient temperature of 17°C or in a portable car fridge for up to 24 h before a fixed-time insemination simplifies the management and insemination protocols for mares and achieves satisfactory embryo recovery rates. Overall, the implementation of this protocol reduces the number of veterinary interventions required for insemination with frozen spermatozoa. In addition, the processing of the spermatozoa and shipping logistics for the frozen–thawed spermatozoa are simplified. Further studies will follow to evaluate different media and refine this protocol for sub-fertile mares and a variety of stallions. Combining the storage of frozen–thawed semen for up to 24 h with fixed-time insemination of mares provides a practical option to simplify frozen semen breeding programmes.

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CONFLICT OF INTEREST STATEMENT

Zamira Gibb is associated with Breed Diagnostics which markets SpermSafe™. Lee Morris is a Director of EquiBreed ART Ltd. Ria Harteveld declares no conflict of interest.

AUTHOR CONTRIBUTIONS

Lee Morris: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; validation; writing – original draft; writing – review and editing. **Ria Harteveld:** Investigation; methodology; writing – original draft. **Zamira Gibb:** Formal analysis; investigation; supervision; validation; writing – review and editing.

DATA INTEGRITY STATEMENT

Lee Morris had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

ETHICAL ANIMAL RESEARCH

Research ethics committee oversight not currently required by this journal: procedures were part of routine breeding management.

INFORMED CONSENT

Owners and agents were aware that excess samples and data from medical records might be used for research in general.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.14096.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request: Open sharing exemption granted by editor for this descriptive clinical report.

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REFERENCES

1. Squires E, Barbacini S, Matthews P, Byers W, Schwenzer K, Steiner J, et al. Retrospective study of factors affecting fertility of fresh, cooled and frozen semen. Equine Vet Educ. 2006;18:96–9.
2. Tanner JC, Barrell GK. Reproductive performance of a cohort of Standardbred mares under a commercial breeding system. Equine Vet J. 2024;56(4):776–85. https://doi.org/10.1111/evj.13989
3. Miller CD. Optimizing the use of frozen-thawed equine semen. Theriogenology. 2008;70:463–8.
4. Sieme H, Bonk A, Hamann H, Klug E, Katila T. Effects of different artificial insemination techniques and sperm doses on fertility of normal mares and mares with abnormal reproductive history. Theriogenology. 2004;62:915–28.
5. Hollinshead FK, Hanlon DW. A single fixed-time insemination prior to ovulation results in higher pregnancy rates than post-ovulation insemination when using frozen-thawed semen. J Equine Vet. 2018;66:198–9.
6. Sabatini C, Rota A, Panzani D, Tesi M, Camillo F. Postmating endometritis and pregnancy rate were not affected by the addition to frozen-thawed semen of filtered seminal plasma when mares without evidence of endometritis were artificially inseminated once 40 hours post-gonadotropin-releasing hormone treatment. J Equine Vet. 2018;62:54–9.
7. Avanzi BR, Ramos Rdos S, Araujo GH, Fioratti EG, Trinca LA, Dell'Aqua JA Jr, et al. Fixed-time insemination with frozen semen in mares: is it suitable for poorly fertile stallions? Theriogenology. 2015;83:1389–93.
8. Morris LH, Tiplady C, Allen WR. Pregnancy rates in mares after a single fixed time hysteroscopic insemination of low numbers of frozen-thawed spermatozoa onto the uterotubal junction. Equine Vet J. 2003;35:197–201.
9. Prell MJ, McCue PM, Moffett PD, Graham JK. Motility and fertility evaluation of thawed frozen stallion semen after 24 hours of cooled storage. J Equine Vet. 2020;90:102983.
10. Luhr J, Oldenhof H, Bigalk J, Martinsson G, Burger D, Sieme H. Evaluation of fertilization capacity of cryopreserved stallion sperm, directly after thawing and after cooled storage. J Equine Vet. 2018;66:72.
11. Clulow J, Gibb Z. Liquid storage of stallion spermatozoa—past, present and future. Anim Reprod Sci. 2022;247:107088.
12. Ricker JV, Linfor JJ, Delfino WJ, Kysar P, Scholtz EL, Tablin F, et al. Equine sperm membrane phase behavior: the effects of lipid-based cryoprotectants. Biol Reprod. 2006;74(2):359–65.
13. Peña FJ, Gibb Z. Oxidative stress and reproductive function: oxidative stress and the long-term storage of horse spermatozoa. Reproduction. 2022;164:F135–44.

14. Vigolo V, Gautier C, Falomo ME, Aurich C. Selection of frozen-thawed stallion semen by microfluidic technology. Reprod Domest Anim. 2023;58:443–9.
15. Gonzalez-Castro RA, Carnevale EM. Use of microfluidics to sort stallion sperm for intracytoplasmic sperm injection. Anim Reprod Sci. 2019;202:1–9.
16. Felix MR, Hinrichs K. Selection of frozen-thawed semen for standard in vitro fertilization. J Equine Vet. 2023;125:104648.
17. Rappa KL, Rodriguez HF, Hakkarainen GC, Anchan RM, Mutter GL, Asghar W. Sperm processing for advanced reproductive technologies: where are we today? Biotechnol Adv. 2016;34:578–87.
18. Cuervo-Arango J, Nivola K, Väihkönen L, Katila T. The effect of storage temperature of stallion semen on pregnancy rates. J Equine Vet. 2015;35:611–6.
19. Hernández-Avilés C, Love CC, Serafini R, Ramírez-Agámez L, Friedrich M, Ghosh S, et al. Effects of glucose concentration in semen extender and storage temperature on stallion sperm quality following long-term cooled storage. Theriogenology. 2020;147:1–9.
20. Morris L. Advanced insemination techniques in mares. Vet Clin North Am Equine Pract. 2006;22:693–703.
21. Squires E. Current reproductive technologies impacting equine embryo production. J Equine Vet. 2020;89:102981.
22. McKinnon AO, Squires EL. Equine embryo transfer. Vet Clin North Am Equine Pract. 1988;4:305–33.
23. Serrano YR, Crabtree JR. Efficacy of deslorelin acetate injection in mares. Equine Vet J. 2019;51(S53):7–8.
24. Kotilainen T, Huhtinen M, Katila T. Sperm-induced leukocytosis in the equine uterus. Theriogenology. 1994;41:629–36.
25. Troedsson MH, Loset K, Alghamdi AM, Dahms B, Crabo BG. Interaction between equine semen and the endometrium: the inflammatory response to semen. Anim Reprod Sci. 2001;68:273–8.
26. Katila T. Effect of the inseminate and the site of insemination on the uterus and pregnancy rates of mares. Anim Reprod Sci. 2005;89:31–8.
27. Güvenc K, Reilas T, Katila T. Effect of insemination dose and site on uterine inflammatory response of mares. Theriogenology. 2005;63:2504–12.
28. Morris LH, McCue P, Aurich C. Equine endometritis: a review of challenges and new approaches. Reproduction. 2020;160:R95–R110.
29. Morris LH, Maclellan LJ. Update on advanced semen-processing technologies and their application for in vitro embryo production in horses. Reprod Fertil Dev. 2019;31:1771–7.
30. Gibb Z, Lambourne SR, Aitken RJ. The paradoxical relationship between stallion fertility and oxidative stress. Biol Reprod. 2014;91:77.
31. Gibb Z, Aitken RJ. The impact of sperm metabolism during in vitro storage: the stallion as a model. Biomed Res Int. 2016;2016:9380609.
32. Gibb Z, Clulow JR, Maclellan LJ, Morris LHA, Aitken RJ, Swegen A. Storing stallion sperm in SpermSafe™ at 17°C may improve fertility by reducing mPTP formation. J Equine Vet. 2023;125:104589.

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