Technique Report

Successful pregnancy using stallion semen stored at 17°C for 6 days

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Abstract

Aim of this study was to assess the effectiveness of Beyond® in maintaining sperm motility at 17°C with 'poor cooler' stallion semen and achieving pregnancy after artificial insemination. An ejaculate was diluted in EquiPlus (Minitube) and divided into 2 aliquots. One was kept at 5°C (control sample), whereas the other was centrifuged, further diluted in Beyond® and kept at 17°C in a semen cooler (Minitube) for 10 days. Total motility (TM) and membrane integrity were assessed daily. For the control sample, TM was 65 and 35% after 24 hours and 48 hours, respectively, whereas the sample diluted with Beyond® still had a TM of 40% after 10 days. Six days after storage at 17°C, a mare was inseminated; a healthy colt was born 344 days after ovulation. We are reporting the first successful pregnancy after storing semen from a poor cooler stallion for 6 days at 17°C.

Keywords: Dilution, extender, stallion, semen, pregnancy

Introduction

Artificial insemination with cooled semen is a common practice in equine reproduction.[1,2] Current commercial extenders normally allow semen doses to be stored at 5°C for up to 48 hours after collection but storage at 15°C is not often practiced. This is due to detrimental side effects, mainly related to bacterial growth at this temperature, and increases in reactive oxygen species production in the sample, in response to higher sperm metabolism.[3,4] Semen survival may vary according to the dilution rate, extender characteristics, and the stallion semen preference for specific formulas.[1,5,6] Recently, a new extender containing antioxidants, antibiotics, and antifungals that allows stallion semen to survive up to 7 days at 17°C after colloid centrifugation, has been described.[7] Field trials using this new type of extender after simple centrifugation with a milk-based extender had good sperm progressive motility.[8] Furthermore, fixed time insemination of frozen-thawed semen after microfluidic selection and storage at 17°C with this extender for 24 hours, produced embryo recovery rates of ~ 50%.[9]

Cooled semen storage, usually performed at 5°C, is a useful strategy in equine breeding management for stallions with restrictions on frequent collection or those that produce a small number of sperm per ejaculation, allowing the storage of new ejaculates until an insemination dose is reached.[10] Semen storage also avoids the need for daily ejaculate splitting in reproduction centers to produce fresh, cooled, and frozen doses. This way, a complete ejaculate can be used to produce cooled doses available throughout the week, allowing subsequent collections to be exclusively used for freezing. Furthermore, semen storage at 15-17°C would be particularly advantageous for stallions that are poor coolers, as their more sensitive membranes are less stable under colder refrigeration.[6,11]

Fresh semen doses for artificial insemination should preferably be available within a 24-hours window before the expected ovulation time, as fertility can be lower in protocols that delay ovulation versus those that induce ovulation.[12,13] Therefore, availability of fresh semen doses is required within a short interval after a preovulatory follicle is identified. If the mare ovulates earlier or if the courier transporting the semen doses is delayed, the ovulation can be missed. Consequently, breeding management could be improved by ensuring the continuous availability of semen doses.

Beyond® (Minitüb GmbH, Tiefenbach, Germany) is the first commercial extender of its kind that allows storage of semen doses for up to 14 days at 17°C, maintaining its quality, according to the manufacturer. Therefore, the use of this

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extender could minimize the partial disposal of ejaculates when all doses are not required within 48 hours after semen collection, reduce the frequency of semen collection, and decrease missed ovulations. Aim of this study was to assess the effectiveness of Beyond® in maintaining the sperm motility of semen kept beyond normal interval from a poor cooler stallion and achieving pregnancy.

Materials and methods

A 5-year Arabian stallion (part of the herd at MK Arabians Stud in Ajman, United Arab Emirates) was used; stallions on the farm are kept according to national and international regulations for the husbandry and care of equids. Based on its reproductive history, the stallion was considered a poor cooler due to its inability to maintain good sperm motility when samples were stored at 5°C for up to 48 hours[14,15] during the previous 2 breeding seasons, with notably worse performance when 15°C storage was attempted.

Following the farm's routine during the breeding season, after properly washing the penis with plain water, the ejaculate of this trained stallion was usually collected on a dummy up to 3 times a week using a properly heated artificial vagina (Missouri model; Minitube, Germany) without lubricant. Artificial vagina was equipped with a disposable plastic inner liner attached to a semen bottle containing a disposable semen filter to separate the gel fraction. These ejaculates were frequently used for inseminations with fresh semen or stored at 5°C for up to 24 hours, and 1 of them was used for this trial.

The sperm total motility (TM) of raw samples was assessed using optical microscopy by an experienced veterinarian, and the concentration was evaluated with a Neubauer chamber. Based on previous cooling tests, a milk-based extender (EquiPlus Combi – Minitube, Germany) was chosen for this ejaculate`s dilution. Thus, an aliquot of the sample was diluted in EquiPlus in 1:1 volume ratio to be further centrifuged (treatment sample), whereas another aliquot was diluted with the same extender to a concentration of 50 x 10^6 sperm/ml (control sample) and kept into an identified tube. After 30 minutes of incubation at 37°C, TM and membrane integrity (eosin-nigrosin test) were assessed.[16] Treatment sample was centrifuged at 600 g for 20 minutes and the supernatant was discarded. The Beyond® extender, prewarmed to 37°C, was added to the sperm pellet to a concentration of 200 x 10^6 cells/ml, and TM and membrane integrity were evaluated. The ready-to-use semen dose was kept at 17°C in an adjustable semen cooler (Minitube, Germany) for 10 days, whereas the control sample was kept at 5°C. An aliquot from each group was taken daily and warmed to 37°C in a water bath before motility and membrane integrity assessment (Table).

After 6 days of cooled storage with daily monitoring, an insemination dose of 18 ml with 70% TM and 50% of membrane integrity, comprising 420 x 10^6 motile sperm (600 x 10^6 total sperm), was used for a postovulatory deep intrauterine insemination in a 13-year fertile Arabian mare. The criteria used to select the mare for the insemination trial, in addition to a good fertility history, included the presence of an active dominant follicle > 35 mm at 32-38 hours after intravenous hCG (1,500 IU; Ovusyn®, Syntex, Vaca Cua, Corrientes, Argentina) treatment, good uterine edema evaluation (assessed by ultrasonography), and absence of a corpus luteum.[17] Transrectal ultrasonography (Mindray Vetus EQ 7) was performed to monitor the estrous cycle, and then on days 1, 14, and 23 after insemination; was also performed monthly, to assess pregnancy.

Six days after semen collection, a mare with a history of normal previous cycle fertility, from the breeding herd that had been monitored for reproductive purposes was used. Mare was induced to ovulate and was designated for this stallion. Mare was inseminated 38 hours after intravenous hCG treatment; a flexible pipette was introduced through the cervix and guided transrectally to the tip of the uterine horn near the ovulation site.

Transrectal ultrasonographic examination on day 1 after insemination revealed no possible excessive uterine reaction due to new extender or semen dose and no evidence of persistent inflammatory process.[18]

Results

Fourteen days after insemination another transrectal examination and ultrasonography were performed; a 22 mm mobile, embryonic vesicle was observed, consistent with the expected size of a 14-day embryo (Figure).[19] On day 23 of pregnancy, embryo's heartbeat was identified; amniotic sac was attached at left uterine horn base (Figure). Fetal sex was diagnosed on day 60 of pregnancy (genital tubercle was close

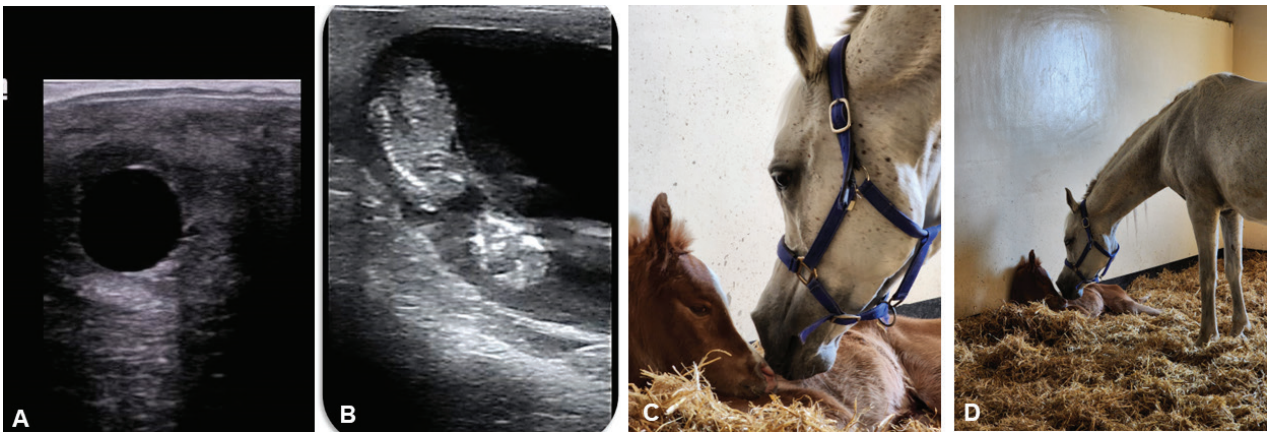

Figure. Embryonic vesicle (A) and embryo (B) at 14 and 60 days after insemination, respectively; healthy colt was recently born (C, D)

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Table. Concentration (sperm x 10^6/ml), total motility (%) and membrane integrity (%) of semen diluted in Beyond (B) or Equiplus (EP) for 10 days

Concentration		EP	B	EP	B
		50	200	50	200
		Total motility		Membrane integrity	
Day	Hours				
0	0	90	85	75	70
1	24	65	85	35	70
2	48	35	85	5	65
3	72	10	80	0	65
4	96	0	80	0	62
5	120	0	70	0	60
6	144	0	70	0	50
7	168	0	65	0	40
8	192	0	60	0	30
9	216	0	50	0	20
10	240	0	40	0	15

to umbilical cord[20]), Progesterone was not supplemented during pregnancy. Parturition occurred on day 344 after ovulation; a healthy colt was born (Figure). Fetal membranes were examined immediately; there were no grossly visible abnormalities.

Discussion

Semen quality and fertility maintenance are affected by the extender composition and its interactions with sperm. Furthermore, temperature, bacterial contamination, and seminal plasma can also influence these parameters.[21] Cooled semen samples have a limited storage period, maintaining good motility and pregnancy rate that normally does not exceed 72 hours.[22,23] A TM range of 30-50% after 72 hours storage at 4°C, using 2 skimmed milk-based extenders (INRA96 and BotuSemen Gold) was reported.[24] In the present study, the TM of sperm diluted in Beyond®, an extender without milk compounds in its formula, also decreased over time but maintained acceptable motility values for up to 10 days of storage.[25] Six days after semen collection, when this sample was used for deep artificial insemination, the TM after warming to 37°C was 70%.

Besides providing an adequate environment and cellular nutrition,[26] a longer sperm storage period might be achieved avoiding oxidation and reducing the membrane damage caused by fluidity changes when the temperature is reduced below 15°C.[6] However, decreasing temperature is a strategy to slow sperm metabolism, controlling reactive oxygen species production and bacterial growth. Therefore, using an extender focused on antioxidation and microbiological control, would allow maintaining semen above 15°C and so reduce sperm damage.[23,27] In the present study, the use of Beyond® allowed semen storage at 17°C, maintaining sperm quality for a longer interval than other protocols that require sample`s cooling at 4-5°C.[24,28] Possibly, other sperm parameters might have been benefited using this extender that remain to be evaluated.

In conclusion, we report the first pregnancy achieved after storing semen for > 48 hours of a poor cooler stallion. Semen can be stored in a new extender (Beyond®) for up to 6 days at 17°C while maintaining sperm fertilization capacity; this opens new possibilities in equine breeding routines, especially for stallions with semen storage difficulties.

Author contributions and agreement

Authors contributions; MN designed, MN conducted, MG analyzed data and wrote the manuscript, and GC and MM involved in manuscript preparation. Authors have read and approved the final version of the manuscript and have agreed to its submission.

Conflict of interest

None to declare.

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