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Evaluation of a cushioned centrifugation technique for processing equine semen for freezing

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Summary
During centrifugation, the use of a cushion associated with a high centrifugation force and clear extenders allows a recovery of 100% of spermatozoa vs 77% for the control. After freezing, the motility and the fertility were similar whatever centrifugation method used.

Introduction

Centrifugation of stallion semen is desirable to eliminate seminal plasma and to increase concentration prior to freezing. The loss of spermatozoa after centrifugation could be a real disadvantage, especially in males producing low number of spermatozoa. Techniques of centrifugation with a cushion, i.e. a denser medium layered on the bottom of centrifuge tube under the extended semen are regularly proposed to improve sperm recovery and/or quality.

Material and Methods

Semen was processed and frozen according to Vidament et al. (2000) except for the steps of first dilution and centrifugation in which various extenders and a cushion (high density solution) were tested. Among the extenders tested, there were one opaque extender containing milk (INRA82 + 2% egg yolk (INRA EY)) and two clear extenders: HGLL+ 1% BSA (HGLL) (Magistrini et al., 1992) and Eqcellsire A + 2% egg yolk (EQ A) (IMV, L'Aigle, France). Antibiotics were added to all these extenders (penicillin 50 000 UI/L and gentamicin 50 mg/L) and the extenders were frozen at -18°C before use.

After collection, spermatozoa were diluted in 50-ml tube to a final volume of 40-45 ml. Semen was centrifuged at 22°C and supernatant and cushion were eliminated, leaving a 3.5 ml pellet. After centrifugation, concentration was adjusted to $100x10^6$ total spermatozoa per ml by addition of freezing extender (INRA EY + 2.5% glycerol) in two steps. Semen was cooled to 4°C for 80 min and frozen in 0.5 ml straws. The cushion was made with Eqcellsire B (EQ B) (IMV). Just before centrifugation, 3.5 ml of EQ B were put slowly under the extended semen and after centrifugation at 1000 g for 20 min, the sperm was found in a concentrated band at the interface of both solutions. The two solutions were removed separately and slowly.

Recovery of spermatozoa after centrifugation was calculated by comparing the number of spermatozoa before and after centrifugation or by comparing the number of produced straws by half ejaculate. After thawing, motility characteristics were evaluated by CASA.

In Exp. 1, 12 split ejaculates were prepared according to 5 treatments (Table 1). Treatment 1 was the control. The treatments 1-4 differed by the extender used for the first dilution and by the presence of cushion. Treatment 5 differed from the others by the ratio of first dilution (15 ml semen, 15 ml extender and 20 ml air) and by the time between collection of semen and the centrifugation (200 min at ambient temperature).

In Exp. 2, ejaculates from 3 stallions were divided in two equal parts before first dilution and centrifugation and processed according to treatments 1 and 4 described in experiment 1. Mares were inseminated with 400 x 10^6 total spermatozoa every day (most often 2 times), with the last AI 24 h before ovulation.

Table 1. Experimental design and results of Experiment 1: sperm recovery after centrifugation and post-thaw motion characteristics [a] (n= 6 stallions x 2 ejaculates).

	Centrifugation treatment				
	1	2	3	4	5
Extender for first dilution	INRA EY	INRA EY	HGLL	EQ A	EQ A
Time collect-centrifugation (min)	20	20	20	20	200
Cushion	/	EQ B	EQ B	EQ B	EQ B
Centrifugation (g x min)	600 x 10	1000 x 20	1000 x 20	1000 x 20	1000 x 20
Recovery sperm (%)[b]	77 b	81 b	103 a	99 a	107 a
Motility (Rapid %) [b]	39	38	40	45	35
Motility (Progressive %) [b]	28 a	27 a	27 a	29 a	20 b
VAP (µm/sec) [b]	73 ab	71 b	80 a	78 ab	79 a

[a] Different letters (a, b) within a row denote differences (P<0.02)
[b] Mean of 6 stallions x 2 ejaculates

Results

In Exp. 1 (Table 1), recovery of sperm was nearly 100% in treatments 3, 4 and 5, and higher than in treatments 1 and 2 (P<0.004). Post-thaw rapid motility was similar among treatments. Progressive motility was less in treatment 5 (P<0.02). Velocity VAP was higher in treatments 3 and 5, but treatment means were similar to control (treatment 1).

In Exp. 2 (Table 2), the number of straws was higher with treatment 4 than with treatment 1 (P<0.04). Post-thaw motility and fertility were similar among treatments.

Discussion

Use of a cushion associated with a clear extender (EQ B or HGLL) improved the recovery rate of spermatozoa after centrifugation in the 2 experiments. Different cushions for centrifugation have been proposed but total sperm recovery was never as high as in this study (Cochran et al., 1984; Volkmann et al., 1987). An increase of around 20-25% in the number of straws produced per ejaculate is really interesting for the equine industry. However, the use of an opaque extender reduces strongly the interest of the cushion.

When semen was centrifuged 20 min after collection, motility was similar among treatments (Exp. 1 and 2) and fertility was similar between the two tested treatments. This demonstrates that this cushion associated with a higher centrifugation force is a safe procedure.

The ability to transport stallion sperm for subsequent freezing at a facility specializing in cryopreservation would be beneficial for equine owners. This is why treatment 5 was included in the experiment 1, with a 3 h delay at ambient temperature before centrifugation and cooling. Although the recovery rate of sperm was nearly 100%, the motility after freezing was

depressed. This was similar to an earlier study (Vidament et al., 2000). The optimal protocol to lengthen the time between collection and freezing has been investigated (Crockett et al., 2001; Backman et al., 2004). A negative interaction between egg yolk and equine seminal plasma during storage at 4°C has been described (Bedford et al., 1995). One explanation for the lower progressive motility observed with treatment 5 could be that this interaction could have taken place during the 3 h delay before centrifugation.

Table 2. Experimental design and results of Experiment 2: number of straws by ejaculate divided in two equal parts at first dilution before centrifugation, post-thaw motion characteristics and per-cycle fertility with frozen semen [a].

	Centrifugation treatment	
	1	4
Extender for first dilution	INRA EY	EQ A
Time collect-centrifugation (min)	20	20
Cushion	No	Yes
Centrifugation (g x min)	600 x 10	1000 x 20
Nb straws / half ejaculate [b]	72 b	92 a
Motility (Rapid %) [b]	54	54
Per-cycle fertility		
stallionG	6 / 8	5 / 8
stallionA	5 / 8	5 / 8
stallionF	4 / 8	5 / 8
Total	15 / 24	15 / 24

[a] Different letters (a, b) within a row denote differences ($P<0.04$)
[b] Mean of 3 stallions x 3 ejaculates

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