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# Use of cushioned centrifugation technique prior to cryopreservation in stallions with good and poor semen freezability

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**Summary**

The work in Experiment I of this study evaluates the effects of routine centrifugation method (10 min., 600 g) vs new techniques for centrifugation (20min., 1000 g) either with or without the use of a cushion fluid (Cushion-Fluid®) on sperm quality postthaw in stallions with good and poor semen freezability (14 stallions: 7 stallions per group x 3 ejaculates) using milk extender for centrifugation. In Experiment II the effects of various centrifugation extenders (Eqcellsire®, INRA-82, HBS) were tested with or without cushioned centrifugation at 1000 xg for 20min (12 stallions: 6 stallions per group x 3 ejaculates). In Exp. I during semen processing increasing gravitational force and prolonged time of centrifugation up to 1000 xg for 20min achieved either by cushioned technique (Cushion-Fluid®) or use of siliconized glass tubes resulted in a more efficient sperm recovery (83%, 92%) when compared to routine method recommended for centrifugation of stallion semen (600 xg, 10min; 75%) ($p<0.05$). After thawing, neither high speed nor prolonged time of centrifugation showed detrimental effects on motility, membrane integrity, acrosomal status, mitochondrial membrane potential, and DNA-integrity of spermatozoa in ejaculates with good and poor freezability when compared to routine centrifugation method. In Exp. II the use of a cushioned centrifugation technique associated with clear-saline centrifugation extender (HBS) or Eqcellsire® resulted in higher sperm recovery (93%, 93%) when compared to INRA-82 (86%) ($p<0.05$). The use of a high-speed centrifugation and clear extender without a cushion allows high sperm recovery (95%) but decreased postthaw sperm quality ($p<0.05$). In summary, high speed and prolonged time cushioned centrifugation of stallion semen prior to cryopreservation needs more operating expenditure than routine centrifugation procedure. The new technique allows higher sperm recovery which is advantageous for the equine industry. Our data also show that post-thaw semen quality is not affected detrimentally provided that milk extender (INRA 82) or Eqcellsire® is used for centrifugation. The study also demonstrates varying effects of extenders on different sperm quality parameters.

**Keywords**: stallion, cryopreservation, semen quality, centrifugation

**Einsatz der "Kissen-Zentrifugationstechnik" vor der Kryokonservierung bei Hengsten mit guter und schlechter Samentiefgefriereignung**

In Experiment I der vorliegenden Arbeit wurden nach Vorverdünnung in Milch-Verdünner (INRA 82) Effekte des routinemäßig eingesetzten Zentrifugationsverfahrens (10 Minuten, 600 xg) im Vergleich zu Zentrifugationsverfahren mit längerer Dauer und höherer Zentrifugalkraft (20 Minuten, 1000 xg) mit und ohne Einsatz einer im Boden des Zentrifugenglas applizierten, nicht-zellpermeabelen Flüssigkeit (Cushion-Fluid®) – sog. „Kissenzentrifugationstechnik" - auf die Qualität des Tiefgefrierspermas von Hengsten mit guter und schlechter Samentiefgefriereignung erfasst (14 Hengste: 7 Hengste/Gruppe, 3 Ejakulate/Hengst). In Experiment II wurden Effekte unterschiedlicher Zentrifugationsverdünner (Eqcellsire®, INRA-82, HBS) mit und ohne Kissenzentrifugation für 20 Minuten bei 1000 xg untersucht (12 Hengste: 6 Hengste/Gruppe, 3 Ejakulate/Hengst). Im Ergebnis (Experiment I) resultierte die Erhöhung von Zentrifugationsdauer und –kraft mit Einsatz der Kissenzentrifugationstechnik oder unter Verwendung von silikonisierten Glasröhrchen ohne Kissen in einer Erhöhung der Spermienrückgewinnungsraten (83%, 92%) im Vergleich zum routinemäßig eingesetztem Verfahren (10 Minuten, 600 xg; 75%) ($p<0.05$). Es ergaben sich keine nachteiligen Effekte auf die Spermienmotilität, das Mitochondrienmembranpotential, die Membran- und Akrosomenintegrität sowie die DNA-Integrität im aufgetauten Samen. In Experiment II wurden nach Vorverdünnung mit salinem Zentrifugationsverdünner (HBS) oder Eqcellsire® hohe Rückgewinnungsraten erzielt (93%, 93%); dieser Unterschied erwies sich als statistisch signifikant im Vergleich zur Kissenzentrifugation mit Milchverdünner (INRA 82: 82%) ($p<0.05$). Die Zentrifugation nach Vorverdünnung mit HBS ohne Kissen ermöglichte hohe Rückgewinnungsraten (95%) aber führte zu einer Beeinträchtigung der Spermaqualität ($p<0.05$). Zusammenfassend bedingt das Kissenzentrifugationsverfahren im Vergleich zum Routineverfahren einen höheren arbeitstechnischen Aufwand; auf der anderen Seite sind die Rückgewinnungsraten bedeutsam erhöht, was wiederum deutliche Vorteile für die Pferde-Besamungszucht liefert. Zudem scheint die Spermaqualität nicht beeinträchtigt, wenn Milchverdünner (INRA 82) oder Eqcellsire® zur Zentrifugation benutzt werden. Des weiteren wurden verdünnerspezifische Einflüsse auf unterschiedliche Spermaparameter festgestellt.

**Schlüsselwörter**: Hengst, Kryokonservierung, Spermaqualität, Zentrifugation

## Introduction

Preceeding cryopreservation of stallion semen centrifugation has become the routine method for removal of seminal plasma. For this purpose, a primary extender is used during centrifugation and a secondary extender is used after centrifugation for dilution and cryoprotection of the sperm rich fraction. Centrifugation may be successfully used, but is not without detrimental effects on motility and morphology of spermatozoa. Despite of the major role of individual composition and

quality of the stallions semen the detrimental effects of centrifugation process can be influenced by temperature, extender, volume, centrifugation time, centrifugation force and volume of supernatant left above pellet (*Pickett* et al. 1975, *Cochran* et al. 1984, *Volkman* et al. 1987, *Heitland* et al. 1996, *Vidament* et al. 2000). Furthermore losses of ~20-40% spermatozoa after centrifugation are disadvantageous, thus it is necessary to evaluate and correct sperm losses after centrifugation to insure proper number of spermatozoa per insemination dose. Different techniques of performing a cushion under the pellet had been developed but they never permitted a total recovery of spermatozoa (*Cochran* et al. 1984, *Volkman* et al. 1987). To enhance sperm recovery after centrifugation, techniques were developed to underlay semen with a dense, liquid cushion, on which the spermatozoa float during the centrifugation process resulting in a total recovery of sperm if clear extender was used (*Revell* et al. 1997, *Ecot* et al. 2005).

The work in Experiment I of this study evaluates the effects of routine centrifugation method (10 min., 600 xg) vs new techniques for centrifugation (20min., 1000 xg) either with or without the use of a cushion fluid (Cushion-Fluid®, Minitüb, Landshut, Germany) on sperm quality postthaw in stallions with good and poor semen freezability. In Experment II the effects of various centrifugation extenders (Eqcellsire®, INRA-82, HBS) were tested with or without cushioned centrifugation at 1000 xg for 20min.

## Materials and Methods

*Animals*

Breeding sires (n=14 in exp. I, n=12 in exp. II) of proven normal fertility were used for the experiment. The stallions belong to the stud farm of the State of Lower Saxony at Celle, Germany, and are routinely used in an AI-program. They participated in the routine semen freezing program, which lasts from October to February. Stallions were divided into 2 groups. Stallions with a progressive post-thaw sperm motility ≤ 35% in more than 3 out of 10 ejaculates collected before the experiment were considered to be poor freezers (n=7 in exp. I, n=6 in exp. II). Stallions above these criteria were assigned to the good freezer group.

*Experimental design*

Semen was collected by artificial vagina on a dummy from each stallion three times per week (Monday, Wednesday, Friday). Sterile gauze filtration sets were used in the collection devices and the gel free semen was evaluated for volume, concentration of spermatozoa by hemocytometer and percentage of progressively motile spermatozoa (pms). Semen was diluted in skim milk extender (INRA 82, *Palmer* 1984) to a final concentration of 50 x $10^6$ spermatozoa/ml and split samples were used to compare centrifugation methods.

In Experiment I sterile plastic centrifugation tubes were filled with 46ml of diluted semen (2.3 x $10^9$ spermatozoa/centrifugation tube) and centrifuged at 600 xg for 10 minutes (control group). A second plastic tube was underlayed with 5 ml of an inert, dense, isotonic solution (Cushion-Fluid®, Minitüb, Landshut, Germany) priot to centrifugation at 1000 xg for 20 minutes (cushion treatment). The third part of the split sample was filled into a sterile, siliconized, conical glass tube and centrifuged at 1000 xg for 20 minutes (glass tube treatment). After centrifugation, supernatant was removed by aspiration (controls and glass tube group) or the sperm rich sperm phase was layered between the interface of cushion-fluid and extender in the supernatant. Supernatant was removed first followed by careful aspiration of the cushion (cushion group).

In Experiment II split ejaculates were prepared in order to compare various centrifugation extenders containing either egg yolk (Eqcellsire® A, IMV, LÁigle, France; group: Eqcellsire®) or milk (group: INRA-82), and a clear-saline extender (Hepes buffered saline, group: HBS). Extenders were added to a final concentration of 50 x $10^6$ spermatozoa/ml. Sterile centrifugation tubes were filled with 46ml of diluted semen (2.3 x $10^9$ spermatozoa/centrifugation tube). The semen was underlayed with 5 ml of an inert, dense, isotonic solution (Cushion-Fluid®, Minitüb, Landshut, Germany; group: INRA-82, HBSC) or with Eqcellsire® B (group: Eqcellsire®) serving as cushion fluid prior to centrifugation at 1000 xg for 20 minutes. Centrifugation of samples diluted in HBS was carried out with (HBSC) and without (HBS) the use of a cushion. After centrifugation, supernatant was removed by aspiration in group HBS or the sperm rich sperm phase was layered between the interface of cushion-fluid and extender in the supernatant and supernatant was removed first followed by careful aspiration of the cushion (group: Eqcellsire®, INRA-82, HBSC).

After resuspending the sperm pellets with milk extender containing 2% egg yolk, sperm concentration was calculated again and freezing extender was added to obtain a final concentration of 200 x $10^6$ spermatozoa/ml and a final concentration of 2.5% glycerol. Semen was equilibrated for 120 min at +5°C packaged in 0.5ml plastic straws and frozen automatically (+5°C to -140°C in 60°C/min.) using a programmable freezer (IMV, LÀigle, France) and were plunged in liquid nitrogen and stored prior to thawing in a waterbath at 37°C for 30sec.

*Sperm assays*

The percentage of progressively motile sperm (PMS) was estimated using a computerized sperm analyzing system (MIKA Motion Analyzer. version 1.1. Mika Medical GmBH, Montreux, Switzerland). Semen samples were diluted with skim milk extender to 25 x $10^6$ sperm/ml and incubated for 2 min prior to analysis at 37°C. Five fields per chamber (MIKA measuring chamber) were analyzed. Sperm kinematics and motility was determined; cells moving slower than 10$\mu$m/sec were considered immotile, whereas cells moving >50$\mu$m/sec were considered to be progressively motile.

Fluorescence stains of spermatozoa were analyzed by flowcytometry (Becton-Dickinson FacsScan, Becton Dickinson Corp., Heidelberg). For each semen sample, 10.000 spermatozoa were differentiated into different cell categories. Plasmamembrane integrity and acrosomal status was evaluated by the FITC-PNA/Syto/PI assay (*Thomas* et al. 1997). Mitochondrial membrane potential was assessed by a modified JC-1 staining procedure (*Gravance* et al. 2000). The integri-

ty of the DNA sperm chromatin structure was measured by using the flowcytometric sperm chromatin structure assay (SCSA™) as described by *Evenson* and *Jost* (2000). The extent of DNA denaturation was expressed in terms of DNA fragmentation index (DFI, formerly termed comp alpha-t), which is the ratio of red to total (red plus green) fluorescence intensity (*Evenson* et al. 2002).

*Statistical analysis*

All percentage data were transformed using arcsin prior to analysis. Data were analyzed by analysis of variance (ANOVA) and means of sperm-treatment were separated using post-hoc comparisons (Student-Newman-Keuls multiple range test). The statistical model were: Experiment I – 2 x 3 factorial, two groups of stallion and 3 centrifugation treatments; Experiment II – 2 x 4 two groups of stallion and 4 centrifugation treatments. Data are expressed as means ± standard deviation. Differences were considered significant at a probability level of 0.05.

**Table 1** Comparison of a routine centrifugation method (10 min., 600 xg) vs. modified techniques for centrifugation (20min., 1000 xg) either with or without the use of a cushion fluid on sperm recovery and post-thaw sperm quality in stallions with good and poor semen freezability using milk extender for centrifugation prior to cryopreservation.
*Vergleich des Routinezentrifugationsverfahrens (10 min., 600 xg) mit modifizierten Zentrifugationstechniken (20min., 1000 xg) bei Vorverdünnung in Magermilchverdünner mit oder ohne Verwendung eines "Kissenmediums" und deren Auswirkung auf die Rückgewinnungsraten und die Spermaqualität nach dem Auftauen bei Hengsten mit guter und schlechter Samentiefgefriereignung.*

| Extender<br>Cushion<br>Centrifug. | Centrifugation techniques<br>Control<br>INRA-82<br>–<br>600 xg, 10min | | Cushion<br>INRA-82<br>Cushion-Fluid®<br>1000 xg, 20min | | Glass tubes<br>INRA-82<br>–<br>1000 xg, 20min | |
|---|---|---|---|---|---|---|
| | Group I | Group II | Group I | Group II | Group I | Group II |
| Sperm recovery | 75,3±2,8 $^{a}$ | 76±1,9 $^{a}$ | 82,9±4,6 $^{b}$ | 83±3,9 $^{b}$ | 92,8±5 $^{c}$ | 92±5,1 $^{c}$ |
| PMS | 51,8±14,8 $^{a}$ | 35,6±13,8 $^{b}$ | 56,4±9,7 $^{a}$ | 36,5±17,7 $^{b}$ | 53,1±12,3 $^{a}$ | 33,9±18,4 $^{b}$ |
| VSL | 37.9±10.8 $^{a}$ | 24.6±11.8 $^{b}$ | 44.1±10.9 $^{a}$ | 28.3±14.6 $^{b}$ | 41.4±11.6 $^{a}$ | 24.4±13.9 $^{b}$ |
| VCL | 96,7±26,3 $^{a}$ | 65,9±24,3 $^{b}$ | 106,8±18 $^{a}$ | 71,9±29$^{b}$ | 95,9±19,9 $^{a}$ | 61,3±27,3 $^{b}$ |
| VAP | 46,9±13,3 $^{a}$ | 31,1±13,2 $^{b}$ | 53,5±11,3 $^{a}$ | 34,4±15,9 $^{b}$ | 49,1±12,1 $^{a}$ | 29,7±15,1 $^{b}$ |
| FITC/PNA-live | 41,5±10,7 $^{ab}$ | 33,2±11,2 $^{c}$ | 49,2±9,5 $^{a}$ | 40,4±12,6 $^{bc}$ | 46,8±5,6 $^{a}$ | 36,1±8,9 $^{bc}$ |
| FITC/PNA-AR | 22,1±8,3 $^{a}$ | 25,1±10$^{a}$ | 19,7±4,8 $^{a}$ | 24,1±8,9 $^{a}$ | 22,1±6,7 $^{a}$ | 21,2±5,5 $^{a}$ |
| JC-1 | 32.9±9.5 $^{a}$ | 26.3±7.4 $^{a}$ | 34.5±8.4 $^{a}$ | 33.0±8.0 $^{a}$ | 34.5±6.6 $^{a}$ | 29.5±7.5 $^{a}$ |
| SCSA/DFI | 15±5,7 $^{a}$ | 17,8±8,6 $^{a}$ | 15,8±5,7 $^{a}$ | 17,5±7,8 $^{a}$ | 16,4±5,2 $^{a}$ | 20,1±8,9 $^{a}$ |

Group: good freezers (group I) ,poor freezers (group II) (n=7 stallions per group; 3 ejaculates/stallion)
PMS: progressively motile sperm (%)
VSL: sperm straight line velocity (µm/sec)
VCL: sperm velocity curvolinear velocity (µm/sec)
VAP: sperm average path velocity (µm/sec)
FITC/PNA-live: Percentage of Syto-positive stained spermatozoa stained by FITC-PNA/Syto/PI.
FITC/PNA-AR: Percentage of FITC-positive stainedacrosomes stained by FITC-PNA/Syto/PI.
JC-1: Percentage of JC-1 positive stained spermmidpiece mitochondrial aggregates.
DFI: denaturation fragmentation index of sperm DNA (%)

$^{a, b, c}$ Values with different superscript differ significantly within rows ($p<0.05$)

## Results

When semen was evaluated after thawing in Experiment I, results indicated that centrifugation of semen extended in INRA-82 at high speed and prolonged time (1000 xg, 20min) allowed recovery of more spermatozoa by the use of cushioned technique (Cushion-Fluid®) or siliconized glass tubes (83%, 92%) when compared to routine method recommended for centrifugation of stallion semen (600 xg, 10min; 75%) ($p<0.05$) (Table 1). As expected, semen samples of the good freezer group had higher PMS, kinematic values (VSL, VAP, VCL) and FITC/PNA-live stained spermatozoa ($p<0.05$), whereas means of FITC/PNA-acrosome stained spermatozoa, JC-1, and DFI where not different between groups ($p>0.05$). Sperm quality parameters did not differ significantly between centrifugation methods ($p>0.05$) (Table 1).

In Experiment II the use of a cushioned centrifugation technique associated with clear-saline centrifugation extender (HBS) or Eqcellsire® resulted in higher sperm recovery (93%, 93%) when compared to INRA-82 (86%) ($p<0.05$) (Table 2). Samples centrifuged with INRA-82 or HBS with cushioned technique had higher PMS and kinematic values when compared to cushioned technique with Eqcellsire® and HBS without the use of a cushion ($p<0.05$). In contrast, values of sperm acrosomal integrity and DNA-integrity were superior for Eqcellsire than for other treated samples ($p<0.05$). The use of high-speed centrifugation and clear extender without a cushion

**Table 2** Comparison of various extenders during high-speed centrifugation (20min., 1000 xg) either with or without the use of a cushion fluid on sperm recovery and post-thaw sperm quality in stallions with good and poor semen freezability.
*Vergleich verschiedener Zentrifugationsverdünner nach Zentrifugation mit erhöhter Dauer und Zentrifugalkraft (20min., 1000 xg) mit oder ohne Verwendung eines "Kissenmediums" und deren Auswirkung auf die Rückgewinnungsraten und die Spermaqualität nach dem Auftauen bei Hengsten mit guter und schlechter Samentiefgefriereignung.*

| Extender<br>Cushion<br>Centrifug. | Centrifugation Techniques<br>Eqcellsire®<br>Eqcellsire® A<br>Eqcellsire® B | | INRA<br>INRA-82<br>Cushion-Fluid® | | HBS+cushion<br>HBS<br>Cushion-Fluid® | | HBS<br>HBS<br>– | |
|---|---|---|---|---|---|---|---|---|
| | 1000 xg 20min | | | | | | | |
| group | I | II | I | II | I | II | I | II |
| Sperm recovery | 93,4$^{a}$ ±5,2 | 93,3$^{a}$ ±5,7 | 85$^{b}$ ±5,9 | 86,4$^{b}$ ±5,4 | 94,5$^{a}$ ±5,7 | 93,1$^{a}$ ±5 | 95,6$^{a}$ ±6,6 | 95,9$^{a}$ ±5,4 |
| Pms | 45$^{bc}$ ±14,5 | 29,3$^{d}$ ±14,6 | 61,1$^{a}$ ±15,7 | 49,8$^{abc}$ ±12,8 | 58,7$^{ab}$ ±15,7 | 46,4$^{abc}$ ±15,7 | 45,1$^{bc}$ ±15,3 | 40,3$^{cd}$ ±16,5 |
| VSL | 29,7$^{bc}$ ±11,8 | 22,2$^{c}$ ±11,6 | 41,1$^{a}$ ±13,1 | 29,4$^{bc}$ ±11,3 | 39,3$^{ab}$ ±14,7 | 30,7$^{abc}$ ±12,6 | 30,1$^{abc}$ ±11,6 | 27,4$^{c}$ ±13,2 |
| VCL | 80,1$^{ab}$ ±2 8 | 63,9$^{b}$ ±26,5 | 102,3$^{a}$ ±23,7 | 82$^{ab}$ ±28,8 | 101,9$^{a}$ ±28,9 | 82,7$^{ab}$ ±26,9 | 77,2$^{b}$ ±23,7 | 70$^{b}$ ±27,3 |
| VAP | 38,2$^{ab}$ ±13, | 28,2$^{b}$ ±12,7 | 50,1$^{a}$ ±13,8 | 36,8$^{ab}$ ±1 3 | 49,4$^{a}$ ±16,4 | 37,7$^{ab}$ ±14,1 | 37,2$^{ab}$ ±13,3 | 31,8$^{b}$ ±13,5 |
| FITC/PNA-live | 40,6$^{ab}$ ±8,7 | 35,2$^{abc}$ ±9,3 | 42,3$^{a}$ ±9,9 | 32,5$^{bc}$ ±8,6 | 43,7$^{a}$ ±6,9 | 33$^{bc}$ ±8,6 | 33,4$^{bc}$ ±8,7 | 29,8$^{c}$ ±9,6 |
| FITC/PNA-AR | 14,9$^{a}$ ±2,7 | 17,2$^{ab}$ ±3,7 | 19,2$^{b}$ ±3,1 | 20,6$^{b}$ ±4,8 | 18,7$^{ab}$ ±3,8 | 20,8$^{b}$ ±6,1 | 20,7$^{b}$ ±3,6 | 21,7$^{b}$ ±5,6 |
| JC-1 | 37,3$^{ab}$ ±6,1 | 32,1$^{bc}$ ±7,4 | 39$^{a}$ ±6,2 | 30,2$^{c}$ ±7,1 | 42$^{a}$ ±6,8 | 32,1$^{bc}$ ±7,4 | 32,2$^{bc}$ ±8,1 | 27,3$^{c}$ ±5,8 |
| SCSA/DFI | 9,7$^{a}$ ±4,6 | 11$^{ab}$ ±4 | 15,7$^{bc}$ ±6,8 | 18,9$^{c}$ ±5 | 13,8$^{abc}$ ±6,3 | 14,6$^{abc}$ ±6,9 | 16,2$^{bc}$ ±8,4 | 14,9$^{abc}$ ±7,3 |

Group: good freezers (group I) ,poor freezers (group II) (n=6 stallions per group; 3 ejaculates/stallion)
Eqcellsire®: package containing centrifugation extender and cushion medium for centrifugation of stallion semen (IMV, Léigle, France)
INRA-82: french skim milk extender
HBS: Hepes buffered saline
Cushion-Fluid®: high density solution for centrifugation of stallion semen (Minitüb, Landshut, Germany)
PMS: progressively motile sperm (%)
VSL: sperm straight line velocity (µm/sec)
VCL: sperm velocity curvolinear velocity (µm/sec)
VAP: sperm average path velocity (µm/sec)
FITC/PNA-live: Percentage of Syto-positive stained spermatozoa stained by FITC-PNA/Syto/PI.
FITC/PNA-AR: Percentage of FITC-positive stained acrosomes stained by FITC-PNA/Syto/PI.
JC-1: Percentage of JC-1 positive stained spermmidpiece mitochondrial aggregates.
DFI: denaturation fragmentation index of sperm DNA (%)

$^{a, b, c}$ Values with different superscript differ significantly within rows ($p<0.05$)

allows highest sperm recovery (95%) but decreased sperm quality postthaw ($p<0.05$).

## Discussion

In equine AI practice stallion semen is generally centrifuged before semen freezing in order to concentrate spermatozoa for further packaging in small plastic straws and to decrease the volume of seminal plasma. Removing the seminal plasma was reported to be necessary for sperm cryosurvival (*Amann* et al. 1987, *Moore* et al. 2005). The centrifugation of spermatozoa at 5°C proved to be less beneficial to spermatozoal motility after 6 to 12 h of storage and then frozen (*Crockett* et al. 2001). *Vidament* et al. (2000) also reported higher motility and fertility if semen was centrifuged at 22° and cooled slowly to 5°C instead of centrifugation at 4°C. In their study, glycerol was added after centrifugation compared to addition of glycerol after cooling immediately. Therefore, in the present study the centrifugation of semen was done immediately following collection, before cooling, storage, and cryopreservation, to ensure higher percentages of motile spermatozoa.

The centrifugation of equine spermatozoa can be detrimental unless low forces are used (*Pickett* et al. 1975). The detrimental effects of the centrifugation process on stallion spermatozoa are mainly influenced by temperature, extender, volume, centrifugation time, centrifugation force and volume of supernatant left above pellet (*Pickett* et al. 1975, *Cochran* et al. 1984, *Volkman* et al. 1987, *Heitland* et al. 1996, *Vidament* et al. 2000). Losses of 20-40% spermatozoa after centrifugation is a real disadvantage, especially in males producing low number of spermatozoa and having a large book of mares. Thus, techniques of high speed centrifugation with a cushion are on demand to improve sperm recovery without affecting sperm quality detrimentally. Our results are in accordance with *Delhomme* et al. (2004). In their study, based on 6 stallions, a 99% recovery rate was obtained, using the cushioned centrifugation technique (Eqcellsire®) compared to 77% recovery for routine centrifugation (600 xg, 10min). However in the present study, recovery rates in Experiment I were not as high when compared to the latter, which might be due to the use of milk-extender instead of a clear saline extender during centrifugation. Similar good results were also obtained, when conical, siliconized glass tubes were used in combination with milk extender for centrifugation; with this method the use of cushioned centrifugation technique can be avoided. A siginificant improvement in sperm recovery was achieved by high speed cushioned centrifugation combined with Eqcellsire® A or clear-saline HBS. It seems that viscosity of these extenders is different in comparison to milk extender and allow a more efficient sperm recovery.

Under the conditions of the present study, higher speed and prolonged time cushioned centrifugation did not affect spermatozoa detrimentally in terms of post-thaw sperm kinematics and motility, membrane integrity, acrosomal status, mitochondrial membrane potential, and spermatozoal DNA integrity. Centrifugation extenders had significant but varying effects on sperm quality in this study. Use of milk extender (INRA-82) resulted in higher values with regard to sperm kinematics and motility postthaw when compared to saline HBS and EqcellsireA; whereas centrifugation with EqcellsireA was followed by a significant improvement of sperm acrosomal integrity and spermatozoal DNA-integrity. The specific composition of EqcellsireA, except that it contains 2% egg-yolk, is not known and protected by the producer. Egg yolk-containing extenders appeared to maintain spermatozoal motility after freezing (*Crockett* et al. 2001) and after centrifugation during cooled storage, especially in stallions considered to be poor coolers (*Brinsko* et al. 2000). On the other hand, egg yolk also was a component of freezing extenders in the present study and is thought to be beneficial to spermatozoal survival during cryopreservation (*Jasko* et al. 1992). *Love* (2005) pointed out that the DNA of stallion sperm that have been collected, processed and handled properly should not show any signs of deterioration, therefore the positive effects of Eqcellsire A serving as a centrifugation extender on spermatozoal DNA-integrity needs further investigation.

*Ecot* et al. (2005) centrifuged split ejaculates either for 10 minutes at 600 xg in INRA 82 or for 20 minutes at 1000 xg in Eqcellsire prior to freezing and inseminated mares with this semen using 400 x $10^6$ total spermatozoa every day, with the last AI 24 h before ovulation. They achieved similar pregnancy rates of 62,5% (15/24).

In summary, high speed and prolonged time cushioned centrifugation of stallion semen prior to cryopreservation needs more operating expenditure than routine centrifugation procedure. The new technique allows higher sperm recovery which is advantageous for the equine industry. Our data also show that post-thaw semen quality is not affected detrimentally provided that milk extender (INRA 82) or Eqcellsire is used for centrifugation. The study also demonstrates varying effects of extenders on different sperm quality parameter.

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