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Effect of slow-cooling, cooled storage, and centrifugation prior to cryopreservation on post-thaw spermatological parameters in stallions classified as good or poor freezers

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Summary: The use of cryopreserved semen has become a standard technique in equine reproduction and is significantly affected by the stallion's individual aptitude for the technique. The aim of the study was to compare the effect of different semen handling protocols before cryopreservation on post-thaw semen quality of stallions (n = 9) either classified as good (n = 5) or poor freezers (n = 4). Ejaculates (n = 6) were collected, splitted and subjected to five different protocols: Group 1 - ejaculates frozen immediately after collection and centrifugation at room temperature (25 °C); Group 2 - ejaculates frozen after cooling at 5 °C for 6 hours with centrifugation before storage; Group 3 - ejaculates frozen after cooling at 5 °C for 6 hours (slow cooling: 0.1 °C/min) without centrifugation before storage; Group 4 - ejaculates frozen after cooling at 5 °C for 24 hours with centrifugation before storage ; Group 5 - ejaculates frozen after cooling at 5 °C for 24 hours (slow cooling: 0.1 °C/min) without centrifugation before storage. Spermatological examination (directly before freezing (T1) and after thawing (T2) included assessment of motility, membrane integrity, acrosomal status and DNA fragmentation index (DFI). Differences between good and bad freezers were observed in all groups. While good freezers had the highest post-thaw progressive motility (T2PM) in Group 1 and 2, poor freezers showed the best results for T2PM in Group 3. Cooled storage for 24 hours resulted in lower T2PM for all groups. Post-thaw DFI and membrane integrity were not affected by treatment group but freezability of the individual stallion. More acrosomal defects were observed in poor freezers as compared to good freezers and after cooled storage for 24 h in poor freezers in comparison to cooled storage for 6 hours. In conclusion, good freezers showed the best post-thaw semen quality after immediate centrifugation and cryopreservation or after centrifugation, followed by cooled storage for 6 hours. Post-thaw semen quality of poor freezers benefitted from slow cooling and cooled storage (6 hours) without previous centrifugation

Keywords: cryopreservation, stallion semen, male heterogeneity, spermatological examination

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Introduction

Although the use of cryopreserved stallion semen increased in recent years, it is still lagging behind the importance of fresh semen (*FN* 2022). However, cryopreserved semen has some undeniable advantages over fresh semen: the possibility of worldwide shipping as well as of employment in a gene reserve program for endangered breeds, the availability of deceased or castrated sires for breeding purposes as well as active competition stallions (*Sieme* 2011). Besides other factors hampering the use of stallions for production of frozen semen (e.g., costs for production of frozen semen and availability of equipment for cryopreservation), one crucial factor is the individual stallion, since not every stallion is a suitable candidate for production of cryopreserved semen (*Aurich* et al. 2020). According to *Tischner*, so called good freezers depict > 40 % progressively motile spermatozoa (PMS), while acceptable or fair freezers contain 20–40 % PMS and bad freezers less than 20 % PMS in a frozen-thawed sample (*Tischner* 1979). *Vidament* et al. estimated that approx. 20 % of stallions can be considered as good freezers, 60 % as fair freezers, and 20 % of stallions produce semen that freezes poorly (*Vidament* et al. 1998). Possible causes for differences in freezability of ejaculates of individual stallions are still not completely elucidated, but age and breed have been determined as explanatory variables in a logistic regression analysis on applicability of ejaculates for cryopreservation (*Aurich* et al. 2020). The mechanisms underlying these differences may be attributed to sperm membrane composition (*García* et al. 2011) and seminal plasma composition (*Aurich* et al. 1996).

A possibility to overcome the need for stallions traveling to semen collection centers where cryopreservation of semen is performed, is the transportation of cooled semen to sites, where cryopreservation equipment is available (*Backman* et al. 2004, *Melo* et al. 2007, *Salazar* et al. 2011). While effects of cryoprotectants and storage protocol (*Melo* et al. 2007), centrifugation technique (*Heutelbeck* et al. 2015), storage

temperature, and sperm concentration (*Backman* et al. 2004) prior to cryopreservation have been evaluated, there is no study examining the effects of the individual freezability of stallions on post-thaw semen quality after cooled storage and subsequent cryopreservation.

Thus, the aim of the study was to compare the effect of different semen handling protocols prior to cryopreservation on post-thaw semen characteristics of stallions either classified as good or poor freezers.

Material and methods

Experimental design

Ejaculates (n = 6) were collected from 9 Hanoverian stallions. Each ejaculate was divided into the following groups: Group 1 - ejaculates frozen immediately after collection and centrifugation at room temperature (25 °C); Group 2 - ejaculates frozen after cooling at 5 °C for 6 hours with centrifugation before storage; Group 3 - ejaculates frozen after cooling at 5 °C for 6 hours (slow cooling: 0.1°C/min) without centrifugation before storage; Group 4 - ejaculates frozen after cooling at 5 °C for 24 hours (slow cooling: 0.1°C/min) with centrifugation before storage ; Group 5 - ejaculates frozen after cooling at 5 °C for 24 hours without centrifugation before storage. Sperm samples were divided into aliquots. One aliquot was diluted and centrifuged, resuspended and immediately frozen. Another aliquot was diluted and centrifuged, resuspended and stored at 5 °C, for 6 (Group 2) and a third aliquot for 24 hours (Group 4) respectively. Both samples were frozen after cooling. The other aliquots were diluted without centrifugation and stored at 5 °C for 6 (Group 3) and 24 hours (Group 5) and later centrifuged and frozen. All samples were diluted and refrigerated using INRA-82 extender. For freezing INRA-82 (incl. 2% egg yolk and 2.5% glycerol) extender was used. Semen samples were evaluated immediately before freezing (T1) and immediately after thawing (T2). A schematic depiction of the study design is made in Fig. 1.

Stallions

Semen was collected from 9 Hanoverian stallions of known fertility. The animals were kept in the semen collection center of the National Stud of Lower Saxony, Celle, Germany and were employed in a commercial breeding program including production of frozen semen. The stallions, aged between 5 and 22 years, were housed in individual stalls on a bed of straw or shavings, fed with oats and hay three times a day, with access to water ad libitum. Handling and husbandry practices were in accordance with national and institutional regulations for the care and use of animals.

Semen collection

Semen, used for the experiments described in this study, was collected from stallions (aged 5–22 years; mean: 10.93 ± 5.03 years) that participated in the routine semen freezing program of the National Stud of Lower Saxony, Celle, Germany. Semen was collected from October to February, during the non-breeding season. Based on previous experiments, stallions were divided into 'good' and 'poor' freezers according to the criteria described by *Vidament* et al. 1998 (*Vidament* et al. 1998): stallions with a post-thaw progressive sperm motility less or equal to 30% in three or more ejaculates out of ten were considered to be poor freezers, whereas stallions exceeding these criteria were designated as good freezers. From each of the 9 stallions (5 'good' and 4 'poor' freezers) six ejaculates were collected. Age of stallions designated as good freezers was 13.6 ± 5.9 years, poor freezers were aged 8.3 ± 5.8 years. Semen was collected every other day using an artificial vagina (Hannover model, Minitube, Tiefenbach, Germany) over a phantom in presence of a mare in heat, and filtered to remove the gel portion.

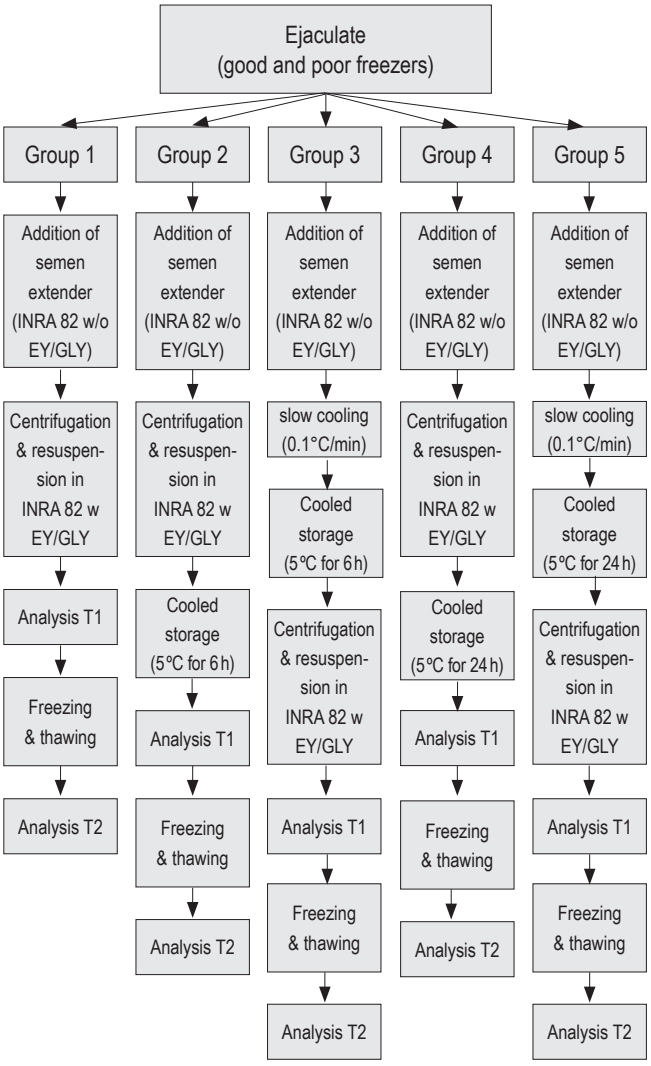

Fig. 1 Schematic depiction of the study design for comparison of possible effects of different semen handling methods (Group 1–5) and aptitude of individual stallions for cryopreservation ("good freezer" vs. "poor freezer") on semen characteristics. Abbreviations: "w/o" = without; "w" = with; "EY" = egg yolk (2.5%); "GLY" = glycerol (2.5%). | *Schematische Darstellung des Studiendesigns zum Vergleich der möglichen Auswirkungen verschiedener Methoden der Samenbehandlung (Gruppe 1–5) und der Samengefriereignung einzelner Hengste („good freezer" vs. „poor freezer") auf die Spermaqualität. Abkürzungen: „w/o" = ohne; „w" = mit; „EY" = Eigelb (2,5%); „GLY" = Glycerin (2,5%).*

Semen processing

Immediately after collection, semen was diluted with INRA-82 at 100×10^6 spermatozoa per mL. Sperm concentration was determined using a Neubauer counting chamber (Kisker, Steinfurt, Germany). INRA-82 extender was prepared by mixing equal amounts of glucose-saline solution and ultra-heat treated skim milk (*Vidament* et al. 2000). INRA-82 consists of: 25 g L^{-1} glucose monohydrate, 1.5 g L^{-1} lactose monohydrate, 1.5 g L^{-1} raffinose pentahydrate, 0.4 g L^{-1} potassium citrate monohydrate, 0.3 g L^{-1} sodium citrate dihydrate, 4.76 g HEPES, pH 7.0, 500 mg L^{-1} penicillin, 500 mg L^{-1} gentamycin, and 0.15 % skim milk. Extended semen was divided into five aliquots as already described. The aliquots were centrifuged either immediately after collection (Group 1, 2, and 4) or stored at 5 °C for 6 hours prior to centrifugation (Group 3) or for 24 hours (Group 4). For Group 5, cooled storage occurred for 24 hours before centrifugation and packaging was performed. Cooled storage (at 5 °C) was done in a tube that was placed in a waterbeaker filled with water at room temperature before cooling down at about 0.1 °C min^{-1} by placing in a cooling cabinet (Minitüb, Tiefenbach, Germany) for 6 hours (Group 3) or 24 hours (Group 5). Aliquots subjected to immediate centrifugation (Group 1, 2, and 4) were packaged into straws at room temperature afterwards and either immediately frozen (Group 1) or stored at 5 °C in a cooling cabinet for 6 (Group 2) or 24 hours (Group 4). Centrifugation was performed at $600 \times g$ for 10 minutes before the resulting sperm pellet was resuspended with INRA-82 supplemented with 2 % clarified egg yolk set to a concentration of 200×10^6 cell mL^{-1}.

Semen freezing

Semen diluted in INRA-82 containing 2 % egg yolk was diluted with an equal amount of INRA-82 containing 2 % egg yolk and 5 % glycerol representing twofold the final desired cryopreservation agent concentration. This resulted in 100×10^6 cells mL^{-1}, and 2.5 % final glycerol concentration. Semen in freezing extender was packaged automatically in 0.5 mL straws (50×10^6 cells per straw) and cooled down to -140 °C at 60 °C min^{-1} using a controlled rate freezer (Minidigitcool; IMV-Technologies, L'Aigle, France) and stored in liquid nitrogen. Straws were thawed in a water bath at 37 °C for 30 seconds after at least 24 h of storage at -196 °C, prior to analysis.

Assessment of sperm motility, membrane, acrosomal and chromatin integrity

Macroscopic parameters (volume, color, consistency, absence of contamination) of the gel-free fraction were assessed. Sperm concentration (per ml) was determined using a Neubauer counting chamber (Kisker, Steinfurt, Germany). By multiplication of volume and sperm concentration, total sperm number (TSN) was calculated.

Semen motility analysis was performed using a phase-contrast microscope (Olympus, Hamburg, Germany) with a stage heater (HT 200, Minitüb, Landshut, Germany) at a temperature of 37 °C combined with a CASA system (CASA: computer-assisted sperm analysis; MIKA Motion Analyser, Windows Version 1.1 Stroemberg, Mika, Montréux, Switzerland). After extension of semen samples and incubation, five fields per chamber (MIKA measuring chamber) were analyzed. Cells moving slower than 10 μm/s were considered immotile, whereas cells moving ≥ 25 μm/s were considered to be progressively motile.

For assessment of pre-freezing as well as post-thaw sperm plasma and acrosome membrane integrity, a staining with SYTO17 (MoBitec, Göttingen, Germany), propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) and fluorescein labeled peanut agglutin (FITC-PNA; Sigma-Aldrich, St. Louis, MO, USA) was performed (*Garner* et al 1999). Stained samples were analyzed using flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany). The flow cytometer was equipped with a 488 nm argon ion laser of 15 mw for excitation, and BP 530/30, BP 582/42 and LP 650 nm filters for green, orange and red fluorescence, respectively. Sperm samples were diluted in 500 μL Tyrode's medium of 300 mOsm kg^{-1} (*Parrish* et al. 1988), at 5×10^6 cells mL^{-1}. Tyrode's medium is composed of: 100 mM NaCl, 3.1 mM KCl, 2.0 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 21.6 mM Na-lactate, 1.0 mM Na-pyruvate, 10 mM HEPES, pH 7.5, 3 g L^{-1} BSA, and 50 μg mL^{-1} gentamicin. Then, 3 μL 3 mM PI, and 5 μL 0.30 mM FITC-PNA were added; resulting in 18 μM PI, and 3 μM FITC-PNA. Samples were incubated for 15 min at 37 °C in darkness, after which green and red fluorescence were detected for 10 000 cells. A flow rate of about 100–300 cells s^{-1} was used. Cells with a plasma membrane that is permeable for PI show red fluorescence of PI bound to DNA, cells with a damaged acrosome show green fluorescence of FITC-PNA bound to glycoproteins, present in the inner site of the outer acrosomal membrane.

Sperm chromosomal integrity was evaluated before as well as after freezing and thawing using the sperm chromatin structure assay (SCSA), in which the denaturability of spermatozoal chromatin is challenged with acid treatment (*Evenson* and *Jost* 2000). In short, samples were diluted in TNE (0.01 M TRIS-HCl, 0.15 M NaCl, 1 mM disodium EDTA, pH 7.4), at approximately 2×10^6 cells mL^{-1}. Thereof, 200 μL were taken and diluted with 400 μL of acid-detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1 % Triton X-100, pH 1.2), and mixed well for 30 seconds. Then, 1.2 mL acridine orange (AO; Polysciences, Warrington, PA, USA) staining solution (0.126 M Na_2HPO_4, 0.037 M citric acid, 0.15 M NaCl, 1 mM disodium EDTA, pH 6.0, containing 6 μg mL^{-1} AO) was added, and the sample incubated in darkness for 3 min at room temperature. Green and red fluorescence emitted by each individual sperm were detected using the same flow cytometer as described above. AO stains intact double-stranded DNA green, and denatured single-stranded DNA red; the acid treatment potentially denatures DNA that is damaged. The DNA defragmentation index (DFI) was calculated from the fractions of cells with single- and double-stranded DNA as described by (*Evenson* and *Jost* 2000). Data analysis was done using 'DAS' (*Beisker* 1994).

Statistical analysis

Analysis of variance was performed using the Statistical Analysis System (SAS) Software Institute, V.9.4, Cary, North Carolina,

USA). The statistical model included fixed effects of treatment (5 groups) and classification either as good or poor freezer on progressive motility (PM), plasma membrane integrity (PMI), acrosomal membrane status, and DNA Fragmentation Index (DFI) before and after cryopreservation. All results are expressed as mean ± standard deviation and p-values < 0.05 were considered statistically significant. Tukey's tests for Post ANOVA Multiple comparisons were used.

Results

Effect of determination as good and poor freezers on semen characteristics

In the present study, spermatological parameters of stallions classified as good and poor freezers for fresh (T1) and thawed (T2) semen samples were evaluated (Table 1). No difference ($p > 0.05$) was seen in stallions from Group 1 (ejaculates frozen immediately after collection) for good or poor freezers for T1PM (progressive motility before freezing: 72.3 ± 14.4% vs. 68.5 ± 12.4%) and T1FITCR2 (membrane and acrosomal integrity before freezing: 50.7 ± 16.3% vs. 48.1 ± 13.4%) respectively, but in relation to all other parameters there was a difference ($p < 0.05$) between good and poor freezers, as shown in Table 1. Regarding Group 2 (ejaculates frozen after centrifugation followed by cooling for 6 hours), no difference ($p > 0.05$) was observed for good or poor freezers in the parameters T1PM (72.2 ± 15.7% vs. 68.6 ± 12.6%), T1FITCR2 (52.8 ± 16.0% vs. 48.1 ± 13.4%), and T1DFI (DNA fragmentation index before freezing: 10.5 ± 4.4% vs. 12.2 ± 4.8%), respectively, but all other parameters differed significantly ($p < 0.05$) between good and poor freezers. In Group 3 (ejaculates frozen after cooling for 6 hours followed by centrifugation), statistical difference was only observed ($p < 0.05$) for T1FITCR2 (56.5 ± 12.9% vs. 40.3 ± 16.0%), while all other parameters remained unaffected. In Group 4 (ejaculates frozen after centrifugation and cooling for 24 hours) the parameters T1FITCR2 (52.4 ± 17.8% vs. 54.8 ± 26.8%) and T2PM (progressive motility after thawing: 19.7 ± 16.7% vs. 16.4 ± 16.0) remained unchanged ($p > 0.05$). Group 5 (ejaculates frozen after cooling for 24 hours followed by centrifugation) showed most variations between good and poor freezers.

Effect of treatment on semen characteristics

T1PM (progressive motility immediately before freezing) was significantly lower in Group 4 as compared to Groups 1–3 (e.g., Group 1 vs. Group 4: 72.38 ± 14.46% vs. 52.45 ± 17.80%; $p < 0.05$) for stallions classified as good freezers (Table 1). No differences among groups were observed for poor freezers. Immediately prior to freezing, T1FITCR2 (membrane and acrosomal integrity) and T1FITCR5 (membrane and acrosomal stained/defect) as well as T1DFI (DNA fragmentation index) did not show differences among groups. For post-thaw progressive motility (T2PM), Group 1 and 2 had significantly higher values as compared to Group 4 and 5 for good freezers (e.g., Group 1 vs. Group 4: 48.39 ± 17.57% vs. 19.73 ± 16.74%). There was also a significant difference in T2PM for good freezers between Group 3 and 4 (37.82 ± 20.22% vs. 19.73 ± 16.74%). For poor freezers, Group 3 displayed higher T2PM values than Group 4 and 5 (e.g., Group 3 vs. Group 4: 33.40 ± 21.21% vs. 16.45 ± 16.00%; $p < 0.05$). No significant difference among groups for good as well as for poor freezers was observed for membrane and acrosomal integrity (T2FITCR2) and DNA fragmentation index (T2DFI) after thawing. While no significant differences were determined for good freezers for the parameter membrane and acrosomal stained/defect (T2FITCR5) after thawing, poor freezers in Group 1 and 2 had higher values as compared to Group 3 and 4 (e.g., Group 1 vs. Group 4: 27.80 ± 7.85% vs. 23.00 ± 6.06%; $p < 0.05$).

Discussion

The aim of the study was to compare the effect of different semen handling protocols prior to cryopreservation on post-thaw semen characteristics of stallions classified either as good or poor freezers.

Classification of stallions as either good or poor freezers was done according to *Vidament* et al. (*Vidament* et al. 1998), setting a threshold value of 30% progressively motile spermatozoa after thawing. This was reflected by the stallions' ejaculates after centrifugation followed by immediate freezing or after cooled storage for 6 hours before freezing. Semen samples stored for 24 hours either with or without centrifugation prior to refrigeration did not exceed a post-thaw progressive motility of 30% at all. This finding was in line with results of a study by *Melo* et al. 2007, where ejaculates were either stored for 24 hours in a cooled equitainer or frozen directly after collection. In this study, none of the examined extenders granted a post-thaw progressive motility above 30%, although no differentiation between poor and good freezers was made (*Melo* et al. 2007). In the present study, post-thaw progressive motility decreased for good freezers, when semen was stored for 6 hours without being centrifuged before (Group 3), as compared to immediate centrifugation and cryopreservation (Group 1) or centrifugation and cooled storage for 6 hours (Group 2). Interestingly, an opposite effect was observed for poor freezers. Here, cooled storage without previous centrifugation resulted in a post-thaw progressive motility value above 30% (33.4 ± 21.2%). Another study investigating the effect of cooled storage with or without centrifugation resulted in lower post-thaw progressive motility, when semen was not subjected to centrifugation prior to cooled storage for 12 hours (*Crockett* et al. 2001). These findings are in line with our results for semen of good freezers stored for 6 hours before centrifugation. However, semen of poor freezers seemed to benefit from cooled storage without previous removal of the seminal plasma. In cattle, a beneficial effect of a prolonged equilibration period (cooled storage without previous centrifugation) on post-thaw progressive motility of semen was found (*Fleisch* et al. 2017). First reported in cattle, a cryoprotective mechanism of protective agents (e.g., egg yolk, milk) through sequestration of bovine seminal plasma proteins by low-density lipoproteins, resulting in increased membrane stability, has been presumed (*Manjunath* et al. 2007). Binder of sperm (BSP) proteins are ubiquitous in seminal plasma among mammals. These proteins fulfill critical functions in the maturation, survival and transport of sperm. Furthermore, BSP proteins can bind to egg yolk low-density lipoproteins and milk com-

Table 1 Comparison of spermatological examination results of stallions classified either as good or poor freezers according to different treatment groups. | *Vergleich der Ergebnisse der spermatologischen Untersuchung von Hengsten mit guter oder schlechter Gefriereigung und eingeteilt in verschiedene Behandlungsgruppen.*

Parameters (in%))	Group 1			Group 2			Group 3			Group 4			Group 5		
	Total	Good Freezers	Poor Freezers	Total	Good Freezers	Poor Freezers	Total	Good Freezers	Poor Freezers	Total	Good Freezers	Poor Freezers	Total	Good Freezers	Poor Freezers
T1PM	70.45 ±13.51	72.38 ±14.46^{a,1}	68.52 ±12.41^{a}	70.46 ±14.25	72.24 ±15.70^{a,1}	68.67 ±12.62^{a}	65.96 ±18.00	67.76 ±15.92^{a,1}	64.15 ±19.96^{a}	53.84 ±23.24	52.45 ±17.80^{a,2}	54.85 ±26.90^{a}	57.60 ±25.22	60.89 ±22.11^{a}	53.74 ±29.10^{a}
T1FITCR2	49.41 ±14.89	50.73 ±16.33^{a}	48.10 ±13.41^{a}	50.48 ±14.86	52.85 ±16.01^{a}	48.10 ±13.41^{a}	48.41 ±16.60	56.51 ±12.94^{a}	40.31 ±16.03^{b}	45.26 ±20.52	55.10 ±16.80^{a}	38.11 ±20.21^{b}	50.42 ±18.49	58.56 ±12.75^{a}	39.55 ±19.64^{b}
T1FITCR5	13.19 ±5,85	11.23 ±4.65^{a}	15.15 ±6.45^{b}	13.25 ±5.80	11.36 ±4.41^{a}	15.15 ±6.45^{b}	12.74 ±4.59	11.66 ±4.77^{a}	13.81 ±4.20^{a}	13.47 ±5.38	10.26 ±3.10^{a}	15.80 ±5.51^{b}	12.52 ±4.59	10.57 ±4.20^{a}	15.12 ±3.81^{b}
T1DFI	11.05 ±4.83	9.80 ±4.53^{a}	12.21 ±4.88^{b}	11.39 ±4.71	10.56 ±4.45^{a}	12.21 ±4.88^{a}	12.22 ±4.37	11.30 ±3.63^{a}	13.16 ±4.88^{a}	10.73 ±4.21	9.41 ±4.15^{a}	11.68 ±4.05^{b}	12.18 ±4.55	11.99 ±4.70^{a}	12.45 ±4.56^{a}
T2PM	35.96 ±20.06	48.39 ±17.57^{a,1}	23.56 ±13.80^{b}	35.68 ±20.94	48.41 ±18.77^{a,1}	22.95 ±14.29^{b}	35.86 ±20.66	37.82 ±20.22$^{a,\#}$	33.40 ±21.21^{a,1}	17.83 ±16.25	19.73 ±16.74^{a,2,‡}	16.45 ±16.00^{a,2}	23.48 ±17.07	27.44 ±15.32^{a,2}	18.20 ±18.25^{a,2}
T2FITCR2	24.74 ±8.42	28.56 ±8.69^{a}	20.52 ±5.63^{b}	23.89 ±8.09	27.26 ±8.13^{a}	20.51 ±5.63^{b}	24.31 ±9.98	25.87 ±10.74^{a}	22.76 ±9.06^{a}	26.56 ±12.05	30.48 ±12.06^{a}	23.71 ±11.39^{b}	29.65 ±11.46	32.41 ±11.01^{a}	25.98 ±11.32^{a}
T2FITCR5	24.94 ±7.61	22.08 ±6.26^{a}	27.80 ±7.85^{b,1}	25.12 ±7.67	22.44 ±6.56^{a}	27.80 ±7.85^{b,1}	21.39 ±6.08	22.48 ±7.04^{a}	20.31 ±4.81^{a,2}	21.57 ±6.42	19.61 ±6.51^{a}	23.00 ±6.06^{b,2}	21.71 ±5.79	21.03 ±6.85^{a}	22.63 ±3.98^{a}
T2DFI	10.87 ±5.07	9.49 ±4.23^{a}	12.24 ±5.35^{b}	10.87 ±5.07	9.49 ±4.45^{a}	12.24 ±5.35^{b}	11.37 ±5.65	10.26 ±4.72^{a}	12.48 ±6.32^{a}	10.87 ±6.12	8.31 ±4.79^{a}	12.74 ±6.37^{b}	11.87 ±6.65	9.59 ±5.00^{a}	14.90 ±7.50^{b}

Table 1 shows the semen evaluation parameters of stallions classified as good and bad freezers for fresh (T1; spermatological examination immediately before cryopreservation) and thawed (T2; spermatological examination immediately after thawing) semen samples. Groups represented the following semen handling protocols: Group 1 - ejaculates frozen immediately after collection and centrifugation at room temperature (25 °C); Group 2 - ejaculates frozen after cooling at 5 °C for 6 hours with centrifugation before storage; Group 3 - ejaculates frozen after cooling at 5 °C for 6 hours without centrifugation before storage; Group 4 - ejaculates frozen after cooling at 5 °C for 24 hours with centrifugation before storage ; Group 5 - ejaculates frozen after cooling at 5 °C for 24 hours without centrifugation before storage. The following parameters were evaluated: progressive motility (PM), membrane integrity (FITCR2), membrane and acrosomal stained/defect (FITCR5), and DNA fragmentation index (DFI). Different letters (a,b) indicate significant differences between good and bad freezers within the same group ($p < 0.05$). Different numbers (1,2) and symbols (#,‡) indicate significant differences among groups ($p < 0.05$) as determined by ANOVA and Tukey's test. All values (in %) are give as mean ± standard deviation. | *Tabelle 1 zeigt die Samenqualitätsparameter von Hengsten mit guter oder schlechter Gefriereignung für flüssigkonservierte (T1; spermatologische Untersuchung unmittelbar vor der Kryokonservierung) und aufgetaute (T2; spermatologische Untersuchung unmittelbar nach dem Auftauen) Samenproben. Die Gruppen entsprachen den folgenden Samenbehandlungsprotokollen: Gruppe 1 - Ejakulate, die unmittelbar nach der Entnahme und Zentrifugation bei Raumtemperatur (25 °C) eingefroren wurden; Gruppe 2 - Ejakulate, die nach 6-stündiger Kühlung bei 5 °C mit Zentrifugation vor der Lagerung eingefroren wurden; Gruppe 3 - Ejakulate, die nach 6-stündiger Kühlung bei 5 °C ohne Zentrifugation vor der Lagerung eingefroren wurden; Gruppe 4 - Ejakulate, die nach 24-stündiger Kühlung bei 5 °C mit Zentrifugation vor der Lagerung eingefroren wurden; Gruppe 5 - Ejakulate, die nach 24-stündiger Kühlung bei 5 °C ohne Zentrifugation vor der Lagerung eingefroren wurden. Die folgenden Parameter wurden bewertet: progressive Motilität (PM), Membranintegrität (FITCR2), Membran- und Akrosomalfärbung/Defekt (FITCR5) und DNA-Fragmentierungsindex (DFI). Unterschiedliche Buchstaben (a, b) zeigen signifikante Unterschiede zwischen Hengsten mit guter und schlechter Gefriereignung innerhalb derselben Gruppe an ($p < 0,05$). Unterschiedliche Zahlen (1,2) und Symbole (#,‡) weisen auf signifikante Unterschiede zwischen den Gruppen hin ($p < 0,05$), ermittelt durch ANOVA und Tukey-Test. Alle Werte (in %) sind als Mittelwert ± Standardabweichung angegeben.*

ponents, an interaction important for the protection of sperm during semen preservation in liquid or frozen state. Our current knowledge of BSP proteins strongly emphasizes their fundamental importance in male fertility and in the optimization of semen preservation techniques (for review: *Plante* et al. 2016). The exact mechanism of BSP has not been elucidated in stallion semen until now, but a beneficial effect of the heat shock protein 2- (HSP-2) and cysteine-rich seminal plasma protein 3 (CRISP-3)-content on the freezability of equine semen has been suggested as stallions with a higher abundance of these proteins depict better post-thawing progressive motility rates (*Jobim* et al. 2011). Although the contents of these proteins were not analyzed in this study, the prolonged equilibration period (6 h) might have compensated the presumably lower concentration of HSP-2 and CRISP-3 in the seminal plasma of poor freezers and therefore aided in stabilization of the spermatozoas' plasma membranes before cryopreservation. This might be supported by the fact, that, in contrast to other groups, no differences in plasma membrane integrity and acrosomal defects post-thawing were observed between good and poor freezers in this group (Group 3). However, the percentage of plasma membrane intact or acrosome-defect sperms did not differ from other groups for poor freezers.

Besides the described effect of the classification as good or poor freezer on the post-thaw motility, additional parameters were influenced by this classification. While the pre-freezing DFI only varied significantly in two groups before freezing, the DFI was significantly higher in post-thaw semen samples of poor freezers in all groups except for Group 3. According to *Love* (2005), a higher DFI is associated with reduced fertility in stallions and can be influenced by semen handling (*Love* 2005). An association of DFI and fertility has also been demonstrated for other species (*Evenson* 2016). Additionally, membrane integrity was found to be higher in most semen samples of good freezers as compared to poor freezers, especially after storage for 24 hours before freezing (Group 4 and 5). Regarding membrane integrity after thawing, good and poor freezers only varied significantly in Group 1, 2, and 4, while the values were nearly identical for Group 3, emphasizing a positive effect of this protocol on post-thaw semen quality of poor freezers. For post-thaw acrosome integrity, a higher percentage of acrosome-defect spermatozoa was observed in poor freezers with the exception of Group 3 and 5. After thawing, semen of poor freezers, either cryopreserved immediately after collection (Group 1) or centrifuged before cooled storage (6 hours, Group 2), depicted a higher percentage of acrosome defect sperms as compared to semen of poor freezers of Group 3. These findings might support the hypothesized beneficial effects of slow cooling (0.1°C/min, followed by storage at 5°C for up to 6 hours followed by centrifugation and packaging at +5°C and freezing) in presence of seminal plasma and egg yolk on membrane stability as described before (*Manjunath* et al. 2007, *Salazar* et al. 2011).

In conclusion, stallions classified as good freezers depicted the best post-thaw semen quality parameters when cryopreservation occurred either immediately after collection and centrifugation or centrifugation and cooled storage (6 hours at 5°C). Post-thaw semen quality of poor freezers benefitted from slow cooling and cooled storage (6 hours) without previous centrifugation. Cooled storage for 24 hours resulted in impaired post-thaw semen samples.

Conflict of interest statement

None of the authors have any conflict of interest to declare.

Author contributions

LB, CM, GM, HS designed the study. CM and LB performed the experiments and semen analysis. LB, MK and AT performed data analysis. LB and MK wrote the manuscript. All authors read, amended and approved the submitted version of the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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