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Cryopreservation of stallion semen: laboratory assessment of sperm injuries after cushioned centrifugation and freezing with conventional and alternative directional freezing methods

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Abstract

Fresh 36 ejaculates of 13 stallions were split into two volumes, centrifuged with and without cushion and frozen with Conventional and two prototype, Drum and Directional, methods using 0.5 ml straws for the Conventional and Drum, and 2 ml flat straws for both the Drum and Directional. Cushioned centrifugation increased total motility (61.2 ± 18.6% vs. 57.5 ± 18.6%; $P < 0.001$) and mean velocity (84.3 ± 15.6% vs. 83.2 ± 13.8%; $P < 0.05$) when compared to not cushioned centrifugation, estimated after cooling the sperm at 4°C for 90 min before freezing. Cushioned centrifugation also increased ($P < 0.001$) spermatozoa with polarized mitochondrial membranes (46.8 ± 11.4% vs. 43.4 ± 10.6%) and intact plasmatic/acrosomal membranes (41.0 ± 11.2% vs. 38.5 ± 11.3%) of frozen/thawed sperm, with respect to not cushioned centrifugation. However, no effects of the centrifugation were evidenced for classical kinetic parameters. Flat straws had negative effect for almost all the parameters analyzed at thawing (T_0) and after 3 hours' incubation at 37°C (T_1), while the Drum method with Paillettes did not show appreciable affects. The variability among stallions was relevant (5% to 69% variance for kinetics and membrane status), while the variability among ejaculates was minor (9% to 28%). Factorial analysis identified three relevant factors with different informational content: Factor 1 represented by membranes status, Factor 2 by kinetics estimated at T_0, and Factor 3 by kinetics estimated at T_1. Cushioned centrifugation had some beneficial effects for the membrane status of the frozen/thawed sperm, while the use of flat straws needs to be improved.

Key Words: cushioned centrifugation, flow cytometer, mitochondrial status, membrane status, kinetics

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Introduction

In equine reproduction the use of cryopreserved semen has rapidly widened in the last decade. Accordingly, the scientific community is increasing its efforts to improve the outcome of this technology of great applicative implications, also considering that other assisted reproductive technologies as sperm sorting, intracytoplasmic sperm injection, and nuclear transfer, although being rarely used, are subject to extensive studies and improvements[12]. In this respect, stallion sperm was frozen with encouraging results using a novel directional freezing method based on multi-thermal gradient[22].

Unlike bulls, stallions are not selected for reproduction on the basis of fertility or sperm freezability, despite there are considerable differences in cryo-tolerance among individuals[17]. Stallion spermatozoa cooled to 1°C undergo a series of phenomena not fully elucidated, which have been collectively termed "cold shock"[1]. The cryodamages include decreased anaerobic glycolysis, mitochondrial membrane integrity and motility[15]. Recently, the intracellular regulating property of sperm survival of the mitochondria during cryopreservation has been partially elucidated[8], and in humans this organelle has been elected as potential marker for sperm fertility[7].

The current freezing protocols for stallion semen involve a dilution into primary extenders, centrifugation for removing seminal plasma and final dilution into freezing extenders. Because centrifugation exerts detrimental effects, a cushioned-centrifugation with a dense liquid cushion has been applied to stallion semen[27]. However, the real beneficial effects of this additional precaution have been questioned[16]. Of considerable interest for the horse breeding industry is the development of effective methods for freezing the sperm in larger volumes than conventional 0.5 ml French straws. This interest stems from the practical use of multiple 0.5 ml straws for stallion spermatozoa, which is still required in AI practice to provide sufficient spermatozoa (up to 800 million) for a breeding dose[21]. However, the handling and manipulation of multiple straws increases this risk of contamination, damage, loss or technical errors. In this respect, cryopreservation in large volumes would enable considerable reduction of such risks and of storage space and costs. Overall, several methods have been described for the packaging of spermatozoa for freezing, which include glass ampoules/vials, polypropylene, polyvinyl or plastic round or flat straws of 0.5–1.0 ml volumes, flat aluminium packets (10–15 ml), pellets (0.1–0.2 ml), and macrotubes[15]. However, results are far from standardized.

Glycerol is the major cryoprotectant routinely used to freeze stallion semen. However, some authors have indicated its capacity of altering the fluidity of plasma membranes[10]. Such a variety of injuries caused by freezing in the stallion sperm emphasize the need for comprehensive diagnostic means to evaluate sperm quality as a whole (vitality, motility, morphology, membranes status, etc.). However, routine methods for sperm analysis are still based only on motility estimates.

The objective of the present work was to monitor a process for sperm cryopreservation, which includes cushioned centrifugation and alternative freezing/packaging methods, through the assessment of both classical kinetics parameters and more advanced evaluation of sperm membrane status in order to assess the potential margins for improvement using standard and experimental approaches.

Material and methods

Sperm collection: Fresh 36 ejaculates were collected from 13 stallions of various breeds (4 to 12 years of age) during the breeding season, using a Missouri-model artificial vagina. After collection, sperm was diluted (1 : 1 v/v) in extender (INRA96, IMV Technologies, L'Aigle, France), shipped to the laboratory at 20°C in thermostatic chamber,

and immediately evaluated for kinetics and membrane status.

Semen processing and freezing: One Conventional and two prototype methods, Drum and Directional, were used for freezing the sperm. Two types of doses were used: a 0.5 ml French straw (Paillette) was used for the Conventional and Drum methods, while a 2 ml flattened shape straw (Flat) (Figure 1), appositely developed for this study, was used for both the Drum and Directional methods.

Fresh ejaculates were frozen as follows: ejaculates were split into two volumes. The first half volume was distributed in 50 ml falcon tubes and the second half was put in falcon tubes in which 8% (v/v) liquid cushion (MaxiFreeze, IMV Technologies) was layered at the bottom. Sperm was then centrifuged at 700 × g for 20 min to eliminate the seminal plasma and extender. Sperm pellets were suspended in the ready to use, egg yolk- and glycerol-containing freezing extender (GENT, 13571/1045, Minitübe International) to a concentration of 100×10^6 sperm/ml in 50 ml Falcon tubes. During processing, sperm was kept at room temperature. After dilution, sperm was sampled and kinetic parameters (C) were recorded. Thereafter, sperm of the two treatments was equilibrated for 90 min at +4°C in refrigerated cabinet. After equilibration, kinetic parameters (E) were recorded on sampled aliquots and sperm was equally loaded into Paillettes and Flats. Sperm was then frozen with Conventional, Drum and Directional methods using the common freezing curve, as follows: +4°C to −20°C at 10°C/minute and −20°C to −140°C at 24°C/minute. Frozen sperm was then stored at −196°C in liquid nitrogen until analysis.

For analysis, sperm was thawed at 37°C for 1 min in water bath and then let stand for 14 min at the same temperature. Thereafter, a first aliquot was analyzed (T_0) for kinetic and membranes status, while the remaining sperm was further incubated at 37°C for 180 min (T_1) and then analyzed for kinetic parameters.

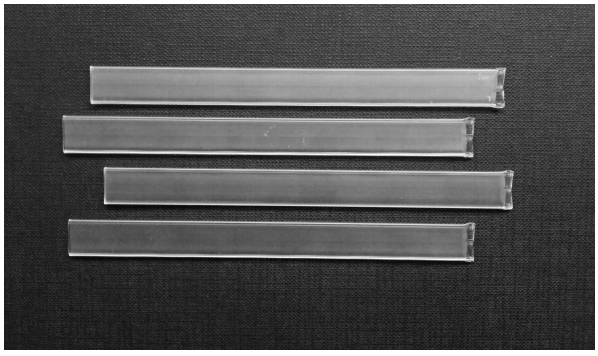

Fig. 1. Experimental alternative 2 ml flat straws of 135 × 12 × 1.65 mm length, width and thickness, respectively.

Freezing equipments: Sperm was frozen using two programmable freezers: Microdigitcool (IMV Technologies) for the Conventional, and a prototype modified Digitcool (IMV Technologies) for the Drum and Directional methods. Differently from the conventional vapor freezing technology, whereas ice grows at an uncontrolled velocity and morphology, the directional freezing techniques allow to control ice crystals propagation using a linear temperature gradient so as to reduce the mechanical damage caused to cells during freezing. This prototype, based on the approach of multi-thermal gradient, is edged by current nitrogen vapor generating a linear gradient system. In the Drum version, as described in the patent (EP2573490A1), a plurality of tubes filled with a predetermined volume of generally defined "biological substance" is placed into a conditioning unit, which is passed through by a flow of nitrogen vapor and simultaneously is driven in rotation so as to obtain a particularly satisfactory heat exchange for the freezing of the conditioning tubes. This prototype can be equipped with a fixed support for Flats straws (Directional) or with a rotating drum (Drum) which supports both the Paillettes and Flat straws.

Sperm analysis:
Standard sperm analysis: Volume was determined using microbalance; total motility, progressive motility and mean velocity were assessed by CASA system (HTM-IVOS vs. 14, Hamilton

Thorne, Beverly, MA, USA), as described: two aliquots of a same sample were layered on two pre-warmed Makler chambers, and at least two microscopic fields were analyzed to count at least 100 sperm per chamber using the following settings: frames per second, 60 Hz; number of frames, 30; cell detection by minimum contrast 10 and minimum cell size 10 pixels. Spermatozoa with average path velocity >15 μm/s were defined as motile, while the motile sperm with a straight-line velocity/average path velocity >0.75 were defined as progressively motile. Concentration and membrane integrity were determined using the NucleoCounter (SP-100™, ChemoMetec, Allerød, Denmark), using the manufacturer's protocol and reagents.

Cytofluorometric sperm analysis: High membrane fluidity, spermatozoa with polarized mitochondrial membranes and plasmatic/acrosomal membrane integrity were determined using the microcapillary flow cytometer (Guava EasyCyte Plus®, IMV Technologies). The instrument is equipped with a 488 nm solid phase blue laser, 2 photodiodes and 3 photomultiplicators (525/30 nm, 583/26 nm, 625/50 nm) able to detect green, orange and red fluorescence. Cytofluorometric data were acquired on a log scale using the CytoSoft software (v S.4.1 beta 5) powered by Guava Technologies. The performance of the flow cytometer was tested daily with the EasyCyte Check kit® (Guava Technologies). Before treatments, fresh and frozen samples were diluted in PBS at a concentration of 30 million sperm/ml. For each assay, approximately 5,000 spermatozoa were analyzed at flow rates of 200–300 cell/sec. Analyses were conducted in duplicates.

Membrane fluidity was evaluated using merocyanine 540 (MC540) as decribed[6]. Briefly, samples were centrifuged for 5 minutes at 160 × g and sperm pellets were suspended in PBS at 30 million sperm/ml. Thereafter, two aliquots were incubated with 2.7 μM MC540 (Molecular Probes Inc., Eugene, OR, USA) for 5 minutes at 37°C in 96-well plates in PBS at sperm concentration of approximately 500 cells/μl. The subpopulation of sperm with minimal red fluorescence (sperm with low membrane fluidity) and the subpopulation with an enhanced red fluorescence (sperm with high membrane fluidity) were discriminated by the red fluorescence histogram.

Spermatozoa with polarized mitochondrial membranes and plasmatic/acrosomal membrane integrity were assessed using the dedicated Easykit 2 (mitochondrial activity; ref: 024864) and Easykit 5 (viability and acrosome integrity; ref: 025293), respectively, using the manufacturer's protocols and reagents (IMV Technologies).

Statistical analysis: At first, data were analyzed using general linear mixed models (GLMM). A first model (model 1) was implemented for evaluating sperm kinetic parameters of fresh sperm assessed after centrifugation (C) and after equilibration (E). Thereafter, two other models (model 2 and 3) were implemented for evaluating sperm measures assessed on the frozen/thawed sperm in order to evaluate respectively: i) effect of the centrifugation regimen (cushion vs. without cushion) on freezing; ii) differences among methods of freezing/packaging. The GLMMs were implemented using sperm measures as dependent variables, Stallion and Ejaculate (nested in Stallion) as random factors, and centrifugation (cushion/without cushion) and freezing/packaging method as the fixed effects. The variance decomposition was obtained for the random effects Stallions and Ejaculates. Finally, the entire dataset of seminal parameters evaluated at T_0 and T_1 was subjected to factorial analysis in order to characterize the informativeness of the measures. More specifically, this statistical approach was used as the classical method for investigating whether a number of variables of interest are linearly related to a smaller number of unobservable factors. Statistical analyses were performed using the R software[20].

Table 1. Kinetic parameters, expressed as mean ± (SD), of fresh sperm (36 ejaculates of 13 stallions) split into two volumes and centrifuged without or with cushion, as assessed immediately after centrifugation (C) and after 90 min' equilibration at +4°C (E)

Centrifugation	TM (C)	TM (E)	PM (C)	PM (E)	MV (C)	MV (E)
Without cushion	68.0 (18.5)	57.5 (18.6)	28.2 (10.4)	26.3 (11.0)	93.5 (16.5)	83.2 (13.8)
With cushion	67.7 (16.6)	61.2 (18.6)***	28.2 (9.7)	26.8 (11.2)	94.2 (14.6)	84.3 (15.6)*

TM (%), total motility; PM (%), progressive motility; MV (μm/sec) mean velocity.
Differences within columns are expressed as: *P < 0.05; **P < 0.01; ***P < 0.001.

Results

Analysis of fresh ejaculates

Results of analysis of fresh ejaculates, expressed as mean ± SD, were as follows: volume = 34.1 ± 14.9 ml; concentration = 424.6 ± 226.7 million/ml; total motility = 77.9 ± 12.0%; progressive motility = 27.8 ± 8.9%; mean velocity = 88.4 ± 14.4 μm/sec; membrane integrity = 82.9 ± 11.7%; high membrane fluidity = 44.5 ± 11.5%; spermatozoa with polarized mitochondrial membranes = 76.9 ± 10.9%; plasmatic/acrosomal membrane integrity = 78.2 ± 6.4%.

Analysis of fresh sperm before freezing

Statistical analysis by GLMM (model 1) of kinetic parameters of fresh sperm assessed immediately after centrifugation (C) did not show significant differences between the cushioned and not cushioned centrifugation (Table 1). However, after 90 min' equilibration at +4°C (E) the cushioned centrifugation revealed significant beneficial effects for total motility ($P < 0.001$) and mean velocity ($P < 0.05$), with respect to the sperm centrifuged without cushion. The statistical analysis evidenced an important variability among stallions for the kinetic parameters (54.5% to 80.8% total variance) (Table 2), while the variability within ejaculates of a same stallion was minor (16.1% to 26.2% total variance).

Analysis of frozen/thawed sperm

The statistical analysis by GLMM (model 2) evidenced a moderate beneficial effect of the cushioned centrifugation ($P < 0.001$) for percentage of spermatozoa with polarized mitochondrial membranes and for spermatozoa with intact plasmatic/acrosomal membranes (Table 3) with respect to the frozen sperm processed without cushioned centrifugation. Likewise, cushioned centrifugation improved membrane integrity although not to a statistically significant level ($P = 0.057$). Differently, beneficial effects of the cushioned centrifugation on the frozen sperm were not revealed by classical computer-assisted kinetic parameters.

With regard to the differences among freezing/packaging methods, statistically evaluated by GLMM (model 3), some major injuries induced by the alternative freezing methods could be evidenced both by classical computer-assisted methods (kinetics) and membranes status as assessed by NucleoCounter and by cytofluorometer (Table 4). In details, with the exception of the Drum method with Paillettes, which did not show statistically significant improvements, the two alternative freezing methods involving the Flat straws had negative effects on sperm for almost all the parameters analyzed at T_0 and T_1, when compared with the traditional freezing method with Paillettes. The statistical analysis evidenced, also in this case, an important variability among stallions, while the variability among ejaculates of the same stallion was minor (Table 2).

With respect to the fresh ejaculates, sperm quality parameters after freezing/thawing showed that, with the exception of the total motility, the kinetics parameters were not indicative of major damages as measured immediately after thawing. Differently, relevant physiological changes of sperm membranes, as evidenced by membrane

Table 2. Random effects Stallion and Ejaculate (nested in Stallion) calculated by decomposition of variance of the General Linear Mixed Models implemented to evaluate fresh sperm analyzed after centrifugation (C) and after 90 min equilibration at +4°C (E), and frozen sperm analyzed at thawing (T_0) and after incubation at 37°C for 180 min (T_1)

		Random effects	
Type of sperm	Variable	Stallion Variance %	Ejaculate Variance %
Fresh	TM (C)	76.1	16.1
	TM (E)	59.9	23.3
	PM (C)	80.8	16.3
	PM (E)	54.5	25.1
	MV (C)	59.9	26.2
	MV (E)	69.9	23.5
Frozen	TM (T_0)	62.7	14.8
	TM (T_1)	45.8	10.2
	PM (T_0)	64.5	15.2
	PM (T_1)	41.2	9.1
	MV (T_0)	46.5	24.6
	MV (T_1)	4.7	27.8
	MI (T_0)	53.3	14.2
	HF (T_0)	49.0	13.4
	POL (T_0)	59.2	11.4
	V+A+ (T_0)	68.9	12.6
	TM (T_0)	62.7	14.8

TM (%), total motility; PM (%), progressive motility; MV (μm/sec) mean velocity; MI (%) membrane integrity; HF (%), high membrane fluidity; POL (%), spermatozoa with polarized mitochondrial membranes; V+A+ (%) spermatozoa with intact plasmatic and acrosomal membranes. Number of ejaculates = 36 (3 for 10 stallions and 2 for 3 stallions).

integrity determined by the NucleoCounter, spermatozoa with high membrane fluidity, spermatozoa with polarized mitochondrial membranes and plasmatic/acrosomal membranes status evaluated by flow cytometry, could be observed.

Factorial analysis

Results of factorial analysis conducted on the entire dataset of seminal parameters relatively to the frozen/thawed semen as estimated at thawing (T_0) and after incubation at 37°C for 180 min (T_1) are presented in Table 5. This statistical analysis allowed to identify three separate relevant factors: Factor 1 representing the state of the membranes with relevant contribute of the parameters high fluidity, membrane integrity, spermatozoa with polarized mitochondrial membranes and plasmatic/acrosomal membrane status, Factor 2 representing sperm kinetics evaluated after incubation (T_1), with relevant contribute of total and progressive motility and Factor 3 representing the kinetics evaluated immediately after thawing (T_0), with major contribute of the variables total and progressive motility and mean velocity.

Discussion

Current laboratory methods for evaluating sperm quality are most entirely based on assessment of kinetic parameters and, particularly in equines, only provide for a partial estimate of fertility[14]. Therefore, because motility assessment

Table 3. Sperm quality parameters, expressed as mean ± (SD), of 36 ejaculates of 13 stallions assessed at thawing (T_0) and after incubation at 37°C for 180 min (T_1). Sperm split into two volumes was centrifuged without or with cushion and frozen with a Conventional and alternative freezing methods

Variable	Without Cushion	With Cushion
TM (T_0)	42.4 (15.9)	40.9 (14.2)
TM (T_1)	13.8 (8.6)	13.2 (8.9)
PM (T_0)	25.3 (9.2)	25.1 (8.7)
PM (T_1)	7.1 (5.3)	8.4 (4.8)
MV (T_0)	75.5 (10.1)	74.8 (9.3)
MV (T_1)	55.7 (10.4)	57.2 (9.8)
MI (T_0)	33.6 (13.5)	37.3 (14.2)
HF (T_0)	79.4 (8.7)	78.2 (9.2)
POL (T_0)	43.4 (10.6)	46.8 (11.4)***
V+A+ (T_0)	38.5 (11.3)	41.0 (11.2)***

TM (%), total motility; PM (%), progressive motility; MV (μm/sec) mean velocity; MI (%) membrane integrity; HF (%), high membrane fluidity; POL (%), spermatozoa with polarized mitochondrial membranes; V+A+ (%) spermatozoa with intact plasmatic and acrosomal membranes.
Differences within rows are expressed as: *P < 0.05; **P < 0.01; ***P < 0.001.

Table 4. Descriptive statistics of sperm quality parameters, expressed as mean ± (SD), of 36 ejaculates of 13 stallions assessed at thawing (T_0) and after incubation at 37°C for 180 min (T_1). Sperm packaged in Paillette or Flat straws was frozen with a Conventional and alternative (Drum and Directional) freezing methods

Variable	Conventional Paillette	Drum Paillette	Drum Flat	Directional Flat
TM (T_0)	41.6 (15.4)	43.7 (17.5)	38.0 (15.2)*	35.7 (16.2)***
TM (T_1)	13.4 (8.0)	15.1 (8.6)	12.7 (9.4)	14.6 (9.7)
PM (T_0)	25.2 (9.9)	25.2 (9.4)	23.4 (8.8)*	22.0 (9.2)***
PM (T_1)	7.3 (4.5)	8.4 (4.7)	7.2 (5.1)	8.7 (6.6)*
MV (T_0)	75.4 (9.9)	74.1 (9.5)	69.2 (9.7)***	70.3 (9.3)***
MV (T_1)	56.3 (8.3)	55.9 (8.4)	51.4 (14.2)**	52.7 (18.0)
MI (T_0)	35.2 (14.8)	38.4 (13.8)	31.3 (13.3)***	33.3 (13.7)
HF (T_0)	78.9 (8.8)	78.1 (9.5)	79.4 (8.8)	79.6 (8.3)
POL (T_0)	44.8 (11.0)	45.7 (10.4)	41.0 (11.5)**	41.3 (12.5)**
V+A+ (T_0)	39.6 (11.3)	40.2 (11.3)	39.3 (12.0)	38.7 (12.1)

TM (%), total motility; PM (%), progressive motility; MV (μm/sec) mean velocity; MI (%) membrane integrity; HF (%), high membrane fluidity; POL (%), spermatozoa with polarized mitochondrial membranes; V+A+ (%) spermatozoa with intact plasmatic and acrosomal membranes.
Means relatively to the columns of Drum and Directional methods differ with respect to the Conventional for: *P < 0.05; **P < 0.01; ***P < 0.001.

is the most immediate and widespread approach among the seminal analysis, in the present work we have evaluated the extension of this routine analysis after incubating the sperm at 37°C, which can be easily adopted in the practice without extra costs. As significant improvement

Table 5. Factorial analysis conducted on the dataset including seminal parameters of 36 ejaculates of 13 stallions evaluated at thawing (T_0) and after incubation at 37°C (T_1) showing three relevant factors with major contribute of the variables in bold type

Parameter	Factor 1	Factor 2	Factor 3
HF (T_0)	**−0.82**	−0.11	
MI (T_0)	**0.68**	0.23	0.37
PM (T_1)		**0.97**	0.22
PM (T_0)	0.28	0.33	**0.86**
TM (T_1)	0.30	**0.85**	0.17
TM (T_0)	0.39	0.32	**0.78**
POL (T_0)	**0.73**	0.21	0.39
V+A+ (T_0)	**0.87**	0.30	0.34
MV (T_1)	0.14	0.34	0.16
MV (T_0)	0.10		**0.54**

TM (%), total motility; PM (%), progressive motility; MV (μm/sec) mean velocity; MI (%) membrane integrity; HF (%), high membrane fluidity; POL (%), spermatozoa with polarized mitochondrial membranes; V+A+ (%) spermatozoa with intact plasmatic and acrosomal membranes.

to the estimate of sperm quality, the use of flow cytometer has enabled a deeper knowledge on sperm status in relation with fertility[9]. In accordance, the results of the present work have shown that assessing the state of membranes by flow cytometry provided additional information, with respect to kinetic parameters, and allowed a more comprehensive evaluation of stallion sperm damages after cryopreservation with different centrifugation and freezing/packaging methods. The major sperm membrane characteristics assessed in the present work will be illustrated in more details.

Sperm plasma membranes are mainly composed of lipids and cholesterol with variable composition among species, individuals, and even ejaculates. These differences among stallions have been related with different levels of fertility and are considered as important markers for stallion sperm adaptability to cooling and freezing[2]. In fact, cooling and cryopreservation may cause changes in sperm plasma membrane, which are potentially responsible for impaired fertility after thawing[19]. The impaired fertility after freezing may be also partially ascribed to an increased proportion of capacitated spermatozoa which, after losing the linear movement, are unable to reach the fallopian tubes[3]. In this regard, in our present work the simple evaluation of kinetic parameters at thawing and after three hours' incubation could reveal a severe decline of sperm motility, as also evidenced by factorial analysis, which highlighted the different informativeness of the same analysis performed at thawing (Factor 3) and after incubation (Factor 2).

Of particular relevance is the evaluation of the portion covering the outer acrosomal membrane, which is physiologically lost during acrosome reaction to allow the release of hydrolytic enzymes. The analysis by cytofluorometer enables the simultaneous assessment of the integrity of both the plasmatic and acrosomal membranes allowing to individuate the spermatic subpopulation with the greatest fertilizing potential. The results of our present work confirm that this parameter largely depends on intrinsic characteristics of individuals, thus representing an important potential marker for individual stallion freezability.

The present study evidenced an increase in sperm population with high membrane fluidity from 44.5% in fresh sperm to approximately 79%

after freezing/thawing with no significant variation among freezing methods, thus indicating a general cryo-injury to membranes that is independent of the freezing methods used. This finding does not surprise as glycerol was specifically indicated as capable of altering sperm membrane fluidity in poultry[10] and swine[4]. In order to better understanding the significance of this measurement it is important to summarize some concepts: after ejaculation, mammalian spermatozoa undergo a series of changes, known as capacitation, which, are characterized by increase in membrane fluidity and acquisition of hypermotility[11]. Many researchers have reported changes after freezing/thawing, which are similar to those occurring during capacitation[25]. This imposes close clinical monitoring of ovulation within the AI practice and increases the number of inseminations required to achieve a pregnancy[24]. It is, therefore, important to monitor these aspects, which are reflected by fluid state of plasma membranes.

Perturbation of mitochondrial function has been found to affect bovine sperm motion[5]. Accordingly, results of our work have shown that the reduction of spermatozoa with polarized mitochondrial membranes (the two methods based on Flat straws) also resulted in the drastic reduction of all the kinetic parameters, while a mechanism of recovery (although only partial) could be observed after three hours of incubation.

The cryodamage associated with the use of Flat straws was relevant. In this regard, it must be mentioned that the freezing methods used in this work were all based on the same freezing curve. Despite the present setting was not advantageous for freezing larger sperm volumes, still this approach offers wide margins for improvement. In this regard, although attempts have been made for freezing stallion spermatozoa in alternative to 0.5 ml straws, results are still controversial[13,18]. However, the varying results, in terms of sperm kinetics and conception rates, are often difficult to compare because of the wide differences in the experiments, methods of sperm analysis that can be either subjective or objective, and the interaction between extender and packaging methods that, altogether, contribute to the differing outcomes. Of great interest, 88% of stallion ejaculates packaged into 12 ml glass cryogenic tubes and frozen by a novel directional freezing technique (MTG® technology)[22] showed visually estimated progressive linear motility $>35\%$ after thawing, which is far higher than we have obtained in the present work with all the tested methods. Moreover, sperm viability and integrity were also significantly superior with respect to the controls frozen by conventional, controlled-rate freezing method. The feasibility of such a method for freezing large volumes was, then, reported on bull sperm[23]. In this case, using the same approach of our present work, sperm kinetic was evaluated using a computer-aided sperm analysis system at thawing and after 3 hours of incubation at 37°C. Taking into account the obvious differences between species and the drastic fall of the kinetic parameters that is even higher than what we have observed, it would be interesting to evaluate the behavior over time even in horse semen to have a more complete picture on how structural sperm damages reflect on motility.

In the past, experiments were designed to optimize cushioned-centrifugation with particular regard to the impact of centrifugation-tube type, composition of the centrifugation medium and centrifugation force on sperm recovery rate and semen quality as assessed immediately after centrifugation and after 24 hours cooled storage[27]. The authors reported that motility of cushion-centrifuged semen was superior with respect to control as assessed after cooling the sperm. In agreement with these authors, in our present work the cushioned centrifugation improved sperm kinetic parameters when assessed after equilibrating the sperm at low temperatures. In addition, in our present work using a iodixanol-based cushion and centrifugation force regimens that were indicated by the same authors as capable of ensuring high sperm harvest, the

beneficial effects of the cushioned centrifugation were also detected after freezing/thawing by more in-depth analysis in cytometry. However, from a more practical point of view, other authors have concluded that layering a cushion solution for centrifugation was both unnecessary and cumbersome for those without considerable experience in the technique[26,16]. The main reason is that, unlike classical density gradient centrifugation protocols, in which sperm are collected in a pellet at the bottom of the tube after the removal of supernatant, when using a cushioned centrifugation, spermatozoa are collected as a layer on top of the cushion. This imposes proper manual skills for the recovery of spermatozoa which can only be achieved with the practice. Therefore, also considering the results of the present work, which have shown what is the extent of improvements, the careful evaluation of the costs/benefits of the cushioned centrifugation should always be conducted in relation to specific requirements and according to the characteristics of individual stallions. Furthermore, given that most difficulties in using liquid cushions increase with small volumes, our experience suggests to process ejaculated in the maximum consented volumes when using cushion centrifugation. This makes it possible to drastically reduce manual difficulty, as we have observed in the present work, allowing to maximize the margins for improvement.

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References

1) Amann, R. P. and Pickett, B. W. 1987. Principles of cryopreservation and review of cryopreservation of stallion spermatozoa. *J. Equine Vet. Sci.*, **7**: 145–173.
2) Aurich, C. 2005. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. *Anim. Reprod. Sci.*, **89**: 65–75.
3) Bailey, J. L., Bilodeau, J. F. and Cormier, N. 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J. Androl.*, **21**: 1–7.
4) Buhr, M. M, Fiser, P., Bailey, J. and Curtis, E. F. 2001. Cryopreservation in different concentrations of glycerol alters boar sperm and their membranes. *J. Androl.*, **22**: 961–969.
5) Ericsson, S. A., Gamer, D. L., Thomas, C. A., Downing, T. W. and Marshall, C. E. 1993. Interrelationships among fluorometric analyses of spermatozoal function, classical semen quality parameters and the fertility of frozen-thawed bovine spermatozoa. *Theriogenology*, **39**: 1009–1024.
6) Gadella, B. M., Harrison. R. A. P. 2000. The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipids transbilayer behavior in the sperm plasma membrane. *Development*, **127**: 2407–2420.
7) Gallon, F., Marchetti, C., Jouy, N. and Marchetti, P. 2006. The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. *Fertil. Steril.*, **86**: 1526–1530.
8) García, B. M., Moran, A. M., Fernández, L. G., Ferrusola, C. O., Rodriguez, A. M., Bolaños, J. M. G., da Silva, C. M. B., Martínez, H. R., Tapia, J. A. and Peña, F. J. 2012. The Mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic-induced stress: role of c-Jun N-terminal kinase (JNK) pathway. *J. Androl.*, **33**: 105–113.
9) Gillan, L., Evans, G. and Maxwell, W. M. C. 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology*, **63**: 445–457.
10) Hammerstedt, R. H. and Graham, J. K. 1992. Cryopreservation of poultry sperm: The enigma of glycerol. *Cryobiology*, **29**: 26–38.
11) Harrison, R. A. P. and Gadella, B. M. 2005. Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology*, **63**: 342–351.
12) Hinrichs, K. 2013. Assisted reproduction techniques in the horse. *Reprod. Fertil. Dev.*, **25**: 80–93.
13) Kozik, D. M., Rubio, C. and Pinto, C. R. 2006. Cryopreservation of stallion spermatozoa in polypropilene cryovial vials. *Anim. Reprod.*

Sci., **94**: 96–98.
14) Kuisma, P., Andersson, M., Koskinen, E., and Katila, T. 2006. Fertility of frozen-thawed stallion semen cannot be predicted by the currently used laboratory methods. *Acta Vet. Scand.*, **48**: 14. (doi:10.1186/1751-0147-48-14.)
15) Lemma, A. 2011. Effect of Cryopreservation on Sperm Quality and Fertility, In: *Artificial Insemination in Farm Animals*, pp. 191–216, Milad Manafi Ed., InTech, Rijeka.
16) Len, J. A., Beehan, D. P., Lyle, S. K. and Eilts, B. E. 2013. Cushioned versus noncushioned centrifugation: Sperm recovery rate and integrity. *Theriogenology*, **80**: 648–653.
17) Loomis, P. R. and Grahamb, J. K. 2008. Commercial semen freezing: Individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. *Anim. Reprod. Sci.*, **105**: 119–128.
18) Lorenzoni, S. L. G., Arruda N. S. and Rodrigues J. L. 2011. Cryopreservation of equine semen loaded in cryovials. *Acta Sci. Vet.*, **39**: 962.
19) Love, C. C., Thompson, J. A., Brinskob, S. P., Rigbyc, S. L., Blanchard, T. L., Lowryd, V. K. and Varner, D. D. 2003. Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. *Theriogenology*, **60**: 1127–1138.
20) R Development Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria 2013. URL http://www.R-project.org/.
21) Samper, J. C. and Morris, C. A. 1998. Current methods for stallion semen cryopreservation: a survey. *Theriogenology*, **49**: 895–903.
22) Saragusty, J., Gacitua, H., Pettit, M. T. and Arav, A. 2007. Directional freezing of equine semen in large volumes. *Reprod. Dom. Anim.*, **42**: 610–615.
23) Saragusty J., Osmers, J. H. and Hildebrandt, T. B. 2016. Controlled ice nucleation—Is it really needed for large-volume sperm cryopreservation? *Theriogenology*, **85**: 1328–1333.
24) Sieme, H. 2009. Semen evaluation. In: *Equine Breeding Management and Artificial Insemination*, pp. 57–70, Ed: J. C. Samper, Saunders Elsevier, Missouri, USA.
25) Thomas, A. D., Meyers, S. A. and Ball, B. A. 2006. Capacitation-like changes in equine spermatozoa following cryopreservation. *Theriogenology*, **65**: 1531–1550.
26) Volkmann D. H. 1987. Acrosomal damage and progressive motility of stallion semen frozen by two methods in 0.5-milliliter straws. *Theriogenology*, **27**: 689–698.
27) Waite, J. A., Love, C. C., Brinsko, S. P., Teague, S. R., Salazar Jr., J. L., Mancill, S. S. and Varner, D. D. 2008. Factors impacting equine sperm recovery rate and quality following cushioned centrifugation. *Theriogenology*, **70**: 704–714.