Centrifugation and addition of glycerol at 22 °C instead of 4 °C improve post-thaw motility and fertility of stallion spermatozoa

Marianne Vidament, P. Ecot, P. Noue, C. Bourgeois, Michèle Magistrini, Eric Palmer

CENTRIFUGATION AND ADDITION OF GLYCEROL AT 22°C INSTEAD OF 4°C IMPROVE POST-THAW MOTILITY AND FERTILITY OF STALLION SPERMATOZOA

M. Vidamentla, P. Ecot2, P. Noue2, C. Bourgeoisl, M. Magistrinil andE. Palmerl

lNational Studs-INRA, PRMD, 37380 Nouzilly, France
2La Jumenterie, National Studs, Cidex 1023bis, Le Pin 61310 Exmes, France

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ABSTRACT

The aims of this study were to evaluate the effects of cooling rate to 4°C and temperature at the time of centrifugation/glycerol-addition (freezing extender: INRA82 + 2% egg yolk + 2.5% glycerol) on postcentrifugation recovery rate, post-thaw motility and per-cycle fertility. When centrifugation/glycerol-addition was performed at 4°C (14 ejaculates), a moderate cooling rate (37°C to 4°C in 1 h) resulted in higher post-thaw motility (45%) than when using a slow cooling rate (37°C to 4°C in 4 h) (39%; P<0.05). When centrifugation/glycerol-addition was performed at 22°C (37°C to 22°C in 10 min) (10 ejaculates), post-thaw motility was lower when spermatozoa were frozen directly from 22°C (23%) than when spermatozoa were cooled to 4°C (22°C to 4°C in 1 h) before freezing (47%; P<0.0001). When centrifugation/glycerol-addition was performed at 22°C (before cooling at a moderate rate), as opposed to 4°C (after cooling at a moderate rate), a significant improvement of 1) recovery of spermatozoa after centrifugation (P<0,0001), 2) post-thaw motility of spermatozoa at thawing (40% vs 36% (n=:;291 ejaculates/group), P<0.0001) and 3) per-cycle fertility (56% vs 42% (n 190 cycles /group), P<0.01) was observed. In conclusion, centrifugation/glycerol-addition at 22°C followed by cooling to 4°C at a moderate rate results in an improvement of post-thaw motility, spermatozoa recovery rate and per cycle fertility.

Keywords: stallion, spermatozoa, cryopreservation, fertility

INTRODUCTION

Sperm freezing ability remains poor for one third of the stallions tested (1, 20, 34). Therefore, the techniques for cryopreservation of equine semen are still in need of further improvement. Common freezing techniques for equine semen include the following steps: dilution of raw semen, cooling to 4°C, centrifugation, addition of freezing extender (containing glycerol and egg yolk), filling straws and freezing. In the literature, the sequence of these steps varies, as does the temperature at which each of these steps are performed (7, 18, 23).

Elimination of seminal plasma is necessary to limit the deleterious effect of seminal plasma on sperm motility during storage (14, 23, 32). Thus, unless one uses only the sperm rich fractions of the ejaculate (31), centrifugation is an obligatory step. In addition to the removal of seminal plasma, the centrifugation step is also necessary for the addition of glycerol extender and the

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a To whom correspondence should be addressed.

adjustment of sperm concentration before freezing (6, 7, 18, 23). Different temperatures at centrifugation have been suggested (18, 23) but only one comparison of the effect of different temperatures on post-thaw motility has been reported. Cochran et al. (7) demonstrated that post-thaw motility was higher when centrifugation was performed at 22°C compared to 37°C. In addition, it has been reported that the percentage of sperm recovered in the pellet after centrifugation varies and is influenced by temperature, gravitational force and centrifugation time (7, 13, 26, 35).

During cooled liquid storage of equine semen, cold-shock damage is a concern and is minimized by cooling the semen at a fixed slow rate from 37 to 4°C (8, 33). Moran et al. (19) demonstrated that stallion spermatozoa are most susceptible to cold-shock between 19°C and 8°C. Thus, the recommended cooling rates for extended semen are: 1) rapid from 37 to 19°C (-0.7 to -2°C/min), 2) slow from 19 to 8°C (-0.05 to -0.1 °C/min) and 3) again rapid from 8 to 4°C (-0.7°C/min) (16, 19). In contrast, the used cooling rate for cryopreservation varies from rapid (20 to 4°C at -10°C/min) to ultra-rapid (20 to 4°C above liquid nitrogen i.e. at -60°C/min) (6, 7, 18).

The aims of the present study were to evaluate 1) the effect of cooling rate from 20 to 4°C (slow: 37 to 4°C in 4 h, -0.2 to -0.06°C/min between 20 and 10°C; moderate: 37 to 4°C in 1 h, -0.8 to -0.3°C/min between 20 and 10°C; or ultra-rapid: 22 to 4°C above liquid nitrogen, -60°C/min) and 2) the effect of temperature and time at which centrifugation/glycerol-addition was performed (at 4°C 1 h after collection; or at 22°C 10 min after collection) on sperm recovery and on post-thaw motility. In addition, the effect of temperature and time of centrifugation/glycerol-addition on fertility was also evaluated. The temperature of 22°C was chosen to ensure that the entire step of centrifugation/glycerol-addition could be done above 19°C, temperature at which cold-shock becomes an important factor.

MATERIALS AND METHODS

General Procedures

Semen collection. Semen was collected from ponies and light-type horses. After collection, the gel-free fraction of the ejaculate was evaluated for volume, sperm concentration and percentage of motile sperm. Only ejaculates with more than 60.10^6 spermatozoa/mL and 50% motile sperm after dilution were used.

Extenders. The basic extender used for these studies was INRA82 (see Table 1). The first dilution and centrifugation were done in INRA82 with 2% centrifuged egg yolk added (EY). After centrifugation, the pellet was reconstituted in INRA82 with 2% centrifuged EY and 2.5% glycerol added. Egg yolk was prepared by centrifugation (600xg 10 min) of fresh egg yolk in INRA82 (v:v 5:4). After EY centrifugation, 90% of the supernatant was aspirated and added to the INRA82 extender to obtain a final concentration of 2% EY.

Freezing and thawing procedure. After collection, sperm was diluted in centrifugation extender (v:v 1:3 to 1:2) in a 50 mL-Corning centrifugation tube to obtain approximately 50.10^6 spermatozoa/mL in a final volume of 45 mL diluted semen. After centrifugation (600xg 10 min), the pellet (3.5 mL) was resuspended in glycerol extender to obtain 100.10^6 spermatozoa/mL.

Temperature at which the centrifugation/glycerol-addition was performed and time of equilibration in glycerol extender differed among the protocols. Protocols were named (Name X/Y) according to temperature at start of centrifugation (X) and temperature at start of freezing (Y) if sperm was not frozen immediately after centrifugation. The protocols are : 1) centrifugation/glycerol-addition at 4°C, followed by 30 to 80 min equilibration at 4°C (4/4); 2)

centrifugation/glycerol-adclition at 22°C, followed by an equilibration during cooling from 22°C to 4°C (22/4) and 3) centrifugation/glycerol-addition at 22°C without equilibration, followed by freezing directly from 22°C (22).

Ali manipulations performed at 4°C were done in a cold-chamber with ail equipment (including centrifuge and straws) precooled to 4°C. Centrifugation at 22°C was performed using a nonrefrigerated centrifuge at room temperature, then glycerol extender maintained at 22°C was added.

Freezing was performed by placing 0.5mL-straws either 10 min at 4 cm above liquid nitrogen or using a programmable freezer (-60°C/min until -140°C), then plunging the straws in liquid nitrogen. Both techniques generate similar freezing rate (7). The same freezing technique was used within a given experiment. At thawing, straws were immersed in a 35 to 37°C water bath for 30 seconds. Motility was analyzed after dilution in 2 mL INRA82 and incubation at 37°C for 10 min. Insemination was performed without dilution or incubation.

Unique aspects of processing (cooling rates from 37°C to 4°C) are described with the individual experiments.

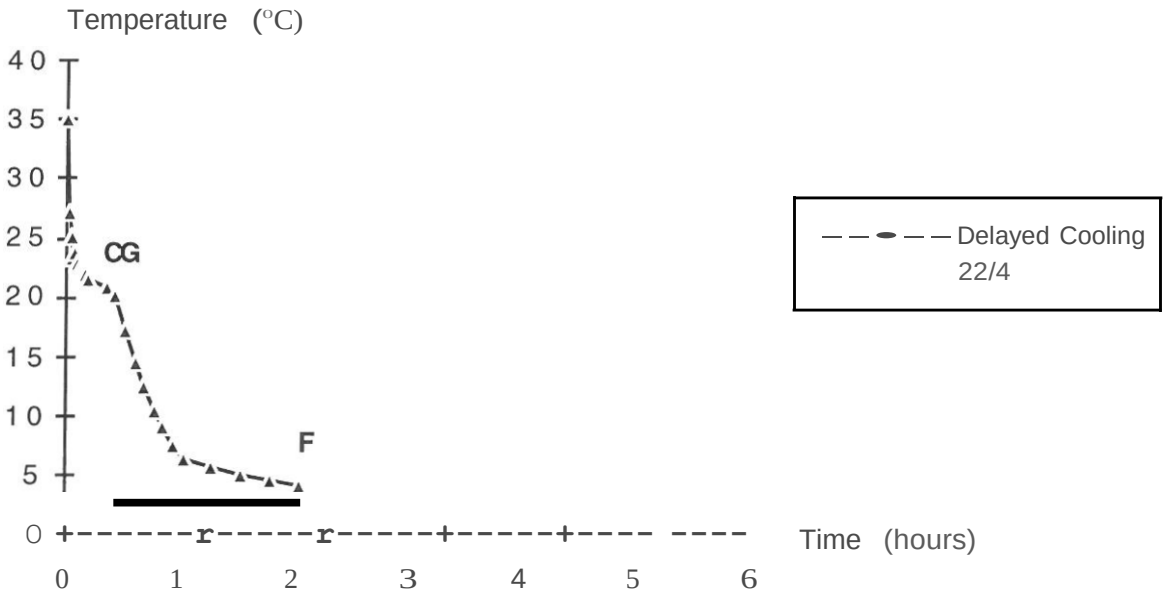
Addition of glycerol and correction of sperm !osses. The entire volume of glycerol extender was added either in one step (no correction of sperm Joss) or in two steps (with correction of sperm loss). The same technique was used within a given experiment. When correction of sperm loss was performed, between 50 and 75% of the final volume of freezing extender was added, concentration was measured using a haemocytometer and the remaining volume was added to obtain the proper concentration (100.106 spermatozoalmL). Correction of sperm Joss extended the time of the centrifugationlglycerol-addition step by 10 to 15 min.

Motility analysis. After thawing, sperm motility was evaluated by CASA (Computer Assisted Sperm Analysis) as described previously (24). Two drops (3 J.LL) per straw and 3 fields per drop were analyzed in a 10 J.LlD depth Makler counting chamber. The average path velocity (VAP), the percentage of total motile spermatozoa (whose VAP was greater than 30 J.Lmls) and the amplitude of lateral head displacement (ALH) were recorded and compared.

Table 1. Composition of the basic extender (INRA 82) with 20 mMoles Hepes (23 modified by 17)

Components		1 liter
Modified HF20		
	Glucose, anhydrous	25 g
	Lactose, 1 H20	1.5g
	Raffinose, 5 H20	1.5g
	Citrate Na, 2 H20	0.25g
	Citrate K, 1 H20	0.41g
	Hepes	4.76g
	Water (apyrogenic)	QSP0.5 L
Skim milk UHT		0.5 L
Gentamicin sulfate		50 mg
Penicillin G		50 000 ur
pH		6.8
müsrnlkg		310

a



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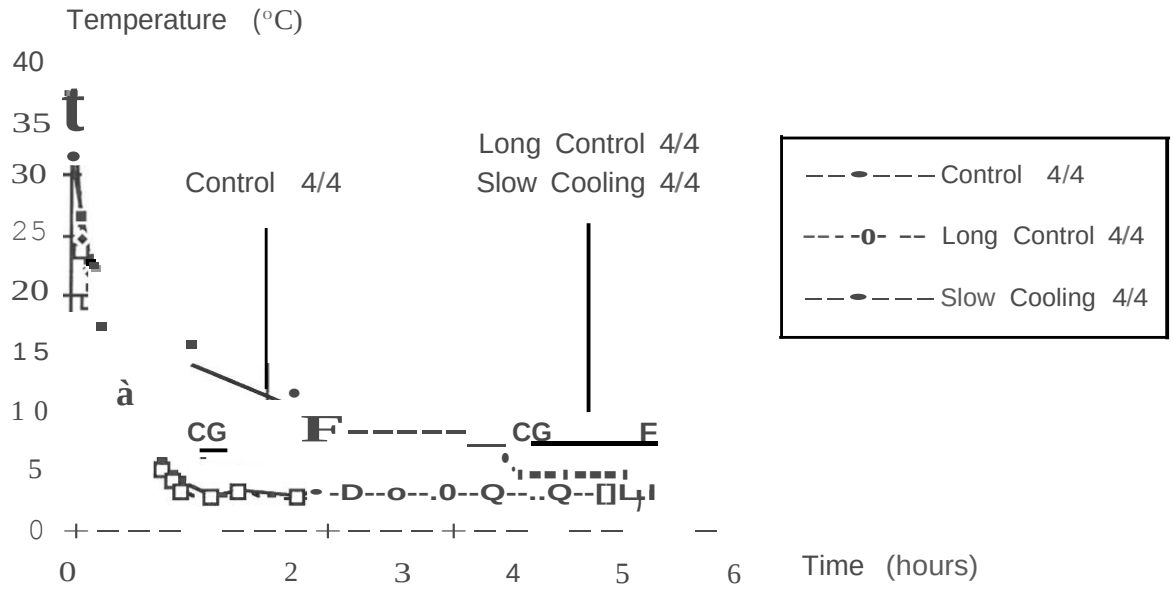

Figure 1. Temperature curves for semen in Experiment 1. First, the protocols had different temperatures and times of centrifugation/glycerol-addition: either at 22°C, 10 minutes after collection (Delayed Cooling 22/4), at 4°C, 1 hour after collection (Control4/4) or at 4°C, 4 hours after collection (Long Control4/4 and Slow Cooling 4/4). Second, the protocols had different cooling rates from 37 to 4°C: moderate in 1 hour (Delayed Cooling 22/4, Control 4/4 and Long Control 4/4) or slow in 4 hours (Slow Cooling 4/4). Freezing occurred at the same time for Delayed Cooling 22/4 and Control4/4 as for Long Control 4/4 and Slow Cooling 4/4.
CG: centrifugation/glycerol-addition F: freezing

Experiment 1

The aims of Experiment 1 were to determine the effect of 1) cooling rate from 37 to 4°C and 2) time and temperature at which centrifugationlglycerol-addition was performed. Fourteen ejaculates (7 stallions x 2 ejaculates) were processed following 4 different protocols with no correction for sperm Joss. After collection and dilution in INRA82 + 2% EY, the semen was divided into 4 aliquots.

The first aliquot (Contro14/4) was processed according to a previously published protocol (23). Semen was cooled from 37°C to 40C in 1 h. The cooling rate was achieved by placing 45 mL diluted semen in a 50 mL tube and placing the semen in a 4°C chamber. The cooling rate was measured using an electronic temperature probe placed in the center of the vial and recorded remotely every 5 min. The cooling rate ranged from -4 to -2°C/rnin between 37 and 22°C, -0.8 to -0.3°C/min between 20 and l0°C, then -0,2°C/min between 10 and 8°C (Figure lb). Subsequentely , the aliquot was centrifuged, and the pellet resuspended in glycerol extender at 4°C. After a 50-min equilibration at 4°C, semen was placed in straws and frozen.

The second aliquot (Long Control 4/4) was prepared and cooled to 4°C in 1 h as described for Control 4/4 but subsequently, the aliquot was maintained for 3 additional hours at 4°C. Centrifugation, pellet resuspension, equilibration, filling straws and freezing were performed as described for Control4/4 (Figure lb).

The third aliquot (Slow Cooling 4/4) was cooled from 37°C to 4°C in 4 h by placing 45 mL diluted semen in a 50 mL tube and immersing the tube in a covered 5-1 glass beaker containing 3 1 water at 20°C in a 4°C chamber. To further control the cooling rate, the lid of the beaker was removed after 2 h. Then, 1 h 50 min later, the 50-mL tube containing the semen was removed from the glass beaker and placed in the 4°C-chamber. The resulting cooling rate was: -4 to -2°C/min between 37 and 22°C, -0.2 to -0.06°C/min between 20 and l0°C, then -0,03°C/min between 10 and 8°C (Figure lb). At the end ofthe cooling procedure, the aliquot was centrifuged, supernatant removed and glycerol extender added. After 50 min equilibration, semen was placed in straws and frozen.

The fourth aliquot (Delayed Cooling 22/4) was placed in a 22°C water bath for 10 min, then centrifuged at room temperature. After resuspension in glycerol extender, sperm was cooled from 22°C to 4°C using the same protocol as described for Control4/4. The resulting cooling rate was: -4 to -2°C/min between 37 and 22°C, -0.4°C/rnin between 20 and 10°C, then -0,2°C/min between 10 and 8°C (Figure la). Then, the semen was maintained at 4°C for 1 h before filling straws and freezing.

For the aliquots Control 4/4 and Long Control 4/4, centrifugation/glycerol-addition was performed at the same interval after collection. For the aliquots Control4/4 and Delayed Cooling 22/4, filling straws and freezing were performed at the same interval after collection. For each ejaculate, five straws per protocol were thawed and evaluated individually for post-thaw motility. The recovery rate of spermatozoa was determined by measuring the concentration in the straws using a haemocytometer.

Experiment 2

The aim of Experiment 2 was to further refine the protocol with centrifugation/glycerol-addition at 22°C (Delayed Cooling 22/4 in Experiment 1) by comparing post-thaw motility after freezing directly from 22°C and freezing after cooling from 22 to 4°C in 1 h. Ten ejaculates (5 stallions x 2 ejaculates) were used. Correction for sperm Joss after centrifugation was included. After collection and dilution, semen was divided into 2 aliquots which were placed in a 22°C waterbath for 10 min, then centrifuged and resuspended in freezing extender, as described above (Delayed Cooling 22/4

in Experiment 1). One aliquot was placed in straws at room temperature and frozen immediately thereafter (Direct Freezing 22). One aliquot was cooled from 22 to 4°C in 1 h before filling straws and freezing (Delayed Cooling 22/4). For each ejaculate, three straws per protocol were evaluated individually for post-thaw motility.

Experiment 3

The aims of Experiment 3 were to confirm the observations made in Experiment 1 on post-thaw motility using the Delayed Cooling 22/4 and Control 4/4 protocols and to compare fertility for these two protocols.

This experiment was performed in four freezing centers of the French National Stud (Haras Nationaux) during 2 winters (1994-95 and 1995-96). For each stallion, three ejaculates were frozen per week and the two protocols were altemated every other week. The protocols are similar to those of Experiment 1 except that time for cooling was longer and time between glycerol addition and freezing varied to accomodate experimental field conditions. In Control 4/4 protocol, semen was cooled to 4°C in 1 h 30 min and equilibrated at 4°C for 30 to 80 min; in Delayed Cooling 22/4 protocol, semen was cooled to 4°C in 1 h 20 min and frozen 0 to 50 min later. The entire ejaculate was frozen in 0.5 mL straws at a final concentration of 100.106 spermatozoalmL since correction for sperm Joss was applied after centrifugation. Recovery of spermatozoa was estimated based on the number of straws produced per ejaculate. The post-thaw motility was evaluated centrally in one laboratory throughout the experimental period on 3 straws per ejaculate. Stallions were included in the comparison of post-thaw motility if more than 2 ejaculates per technique were frozen in the subsequent week.

The frozen semen was used during 3 breeding seasons (1995, 96 and 97) in 40 different insemination centers without knowledge of the freezing protocol. Only ejaculates with post-thaw motility higher than 35% were used for insemination. Mares were first inseminated when in estrus with a follicle larger than 35 to 40 mm. Mares were inseminated with 400.106 total spermatozoa and insemination was repeated every 24 h until ovulation was detected (34). Most mares were inseminated at least 2 times during the same estrus. They were examined for pregnancy between 13 and 35 days postovulation. Only stallions with fertility data for 6 or more cycles per protocol were included in the fertility analysis.

Statistical Analysis

For Experiments 1 and 2, the model was balanced. General differences between means were tested by ANOVA (Anova procedure of SAS; SAS lnstitute Inc., Cary, NC, USA) considering effects of protocol, stallion, ejaculate within stallion and taking the interaction ejaculate by protocol as the error term, after checking that the interaction stallion by protocol was not significant. Means were separated by Student Neumans Keul's test.

For Experiment 3, the mode! was an unbalanced comparison of two protocols between ejaculates within stallion. General differences between means were tested by a factorial analysis for unbalanced data (Glm procedure of SAS) considering effects of protocol, stallion and taking the interaction stallion by protocol as the error term. Differences between percentages (per-cycle fertility) were tested with a Chi-square test. Statistical significance was considered at $P<0.05$.

RESULTS

Experiment 1

The post-thaw motility was higher for the Delayed Cooling 22/4 protocol than for the three other protocols ($P< 0.02$) (Table 2). Among the three 4/4 protocols, post-thaw motility was

Table 2. Post-thaw motion characteristics and recovery rate for Experiment 1. First, the protocols had different temperatures and times of centrifugationlglycerol-addition: either at 22°C, 10 minutes after collection (Delayed Cooling 22/4), at 4°C, 1 hour after collection (Control 4/4) or at 4°C, 4 hours after collection (Long Control 4/4 and Slow Cooling 4/4). Second, the protocols had different rates of cooling from 37 to 4°C: moderate in 1 hour (Delayed Cooling 22/4, Control 4/4 and Long Control 4/4) or slow in 4 hours (Slow Cooling 4/4). Freezing occurred at the same time for Delayed Cooling 22/4 and Control 4/4 as for Long Control 4/4 and Slow Cooling 4/4.

	22/4 Delayed Cooling	Control	4/4 Slow Cooling	Long Control	pooled SEM
Motility (%)	51 a	45 b	39 c	42 be	3
VAP (J.!rniS)	79	78	79	80	2
ALH (J.!m)		4.5 a		4.5 a	0.2
Recovery of spermatozoa after centrifugation measured by concentration in straws (106 spermatozoalml)	92 a	67 b	60 b		5

Values are means of 14 split ejaculates (7 stallions x 2 ejaculates).

abc Different superscripts within a row denote differences (P<0.02)

Table 3. Post-thaw motion characteristics and recovery rate for Experiment 3 (winter 1994-1995). The protocols had different temperatures and times of centrifugationlglycerol-addition: either at 22°C, 10 minutes after collection (Delayed Cooling 22/4) or at 4°C, 1 hour 30 minutes after collection (Control 4/4).

	Delayed Cooling 22/4	Control4/4	pooled SEM
Motility (%)			1
Recovery of spermatozoa after centrifugation (measured by the number of straws/ejaculate)	110 a	92 b	4

Values are least-square means of 131 ejaculates (Delayed Cooling 22/4) and of 114 ejaculates (Control4/4) from 25 stallions.

a b Different superscripts within a row denote differences (P<0.005)

Table 4. Post-thaw motion characteristics and recovery rate for Experiment 3 (winter 1995-1996). The protocols had different temperatures and times of centrifugation/glycerol-addition: either at 22°C, 10 minutes after collection (Delayed Cooling 22/4) or at 4°C, 1 hour 30 minutes after collection (Control4/4).

	Delayed Cooling 22/4	Control4/4	pooled SEM
Motility (%)			
Recovery of spermatozoa after centrifugation (measured by the number of straws/ejaculate)	115 a	97 b	4

Values are !east-square means of 302 ejaculates (Delayed Cooling 22/4) and of 291 ejaculates (Control 4/4) from 55 sta11ions.

ab Different superscripts within a row denote differences (P<0.0001)

significantly less in the Slow Cooling 4/4 than in the Control 4/4 protocol (P<0.02). Post-thaw motility was not different for Control 4/4 and for Long Control 4/4 protocols. VAP did not differ among protocols (Table 2). ALH was similar for ali protocols except for Slow Cooling 4/4 in which it was lower (P< 0.02) (Table 2).Recovery of spermatozoa, as measured by concentration, was higher in the Delayed Cooling 22/4 protocol than in the other protocols (P<O.Oll). For the three 4/4 protocols, recovery was similar.

Experiment 2

Mean post-thaw motility (%) was higher for the Delayed Cooling 22/4 protocol (47 ± 2) than for the Direct Freezing 22 protocol (23 ± 2) (mean± pooled sem) (P <0.0001) (no Table). The velocity VAP was lower for the Delayed Cooling 22/4 protocol than for the Direct Freezing 22 protocol (respectively, 80.5 *11m/s* ± 1.4 vs 84.1 ± 1.4 , P< 0.05). No difference was observed between the two protocols for ALH (4.8 !!ffi± 0.1 vs 4.8 ± 0.1).

Experiment 3

The number of stallions selected for the comparison of post-thaw motility was 27 and 55 for the 2 freezing periods (1994-95 and 1995-96), respectively, and each period was analyzed separately. Similar results were observed during the 2 periods (Tables 3 and 4). Post-thaw motility was significantly higher when semen was processed using the Delayed Cooling 22/4 method compared to the Control 4/4 method (P<0.005). Recovery of spermatozoa, based on number of processed straws per ejaculate, was higher in the Delayed Cooling 22/4 protocol (P<O.OOI).

Thirteen stallions were selected for the fertility comparison. Fertility per cycle was higher when semen was frozen using the Delayed Cooling 22/4 protocol (P<O.Ol) (Table 5).

Table 5. Per-cycle fertility for 13 stallions in Experiment 3. Semen from each stallion was frozen according to two protocols and used for 6 cycles or more over 3 years. The protocols had different temperatures and times of centrifugation/glycerol-addition: either at 22°C, 10 minutes after collection (Delayed Cooling 22/4) or at 4°C, 1 hour 30 minutes after collection (Control4/4)

	Delayed Cooling 22/4		Control4/4	
StaJJions	Fertility per cycle (%)	Number of cycles (n)	Fertility per cycle (%)	Number of cycles (n)
A	85	13	30	10
B	56	9	36	Il
C	63	8	67	6
D	31	16	33	9
E	67	12	22	9
F	47	17	63	8
G	58	83	46	67
H	86	7	11	9
I	45	11	63	16
J	54	39	38	13
K	50	6	33	12
L	56	16	36	14
M	17	6	50	6
TOTAL		243		190

Values are percentages (pregnant cycles *1* (pregnant cycles+ non pregnant cycles))
ab Different superscripts within the row denote differences (P<0.01)

DISCUSSION

In Experiment 1, we observed that post-thaw motility was improved when centrifugation/glycerol-addition was performed at 22°C instead of 4°C. When centrifugation/glycerol-addition was performed at 4°C, post-thaw motility was higher when cooling from 37 to 4°C was done in 1 h compared to 4 h; and sperm recovery after centrifugation was improved when centrifugation/glycerol-addition was performed at 22°C. Subsequently in Experiment 2, we demonstrated that after centrifugation at 22°C, direct freezing from 22°C resulted in a lower post-thaw motility compared with cooling to 4°C before freezing. In Experiment 3, using a more significant number of stallions, we confirmed that centrifugation/glycerol-addition at 22°C resulted in higher post-thaw motility and sperm recovery and in higher per cycle fertility.

Differences in membrane structure and metabolism for spermatozoa maintained at ambient temperature and 4°C could account in part for our observations. Previously it has been reported that in stalJions, spermatozoal membrane lipids are still in liquid phase at 22°C but are in gel phase at 4°C (25). So this phase change could result in increased damage to the sperm cells when they undergo membrane stresses at temperatures below 22°C. ln bulls, metaboJism of spermatozoa is greatly reduced at 21°C (10 to 20% of the metabolism at 37°C) and almost completely reduced at 4°C (2 to 7%)(28). Stallion spermatozoa seem to use different pathways of metabolism in function of the storage temperature: oxidative at 37°C and 15°C and nonoxidative at 4°C (17).

In our study, the recovery rate of spermatozoa was consistently higher after centrifugation at 22°C than at 4°C. The recovery rate of spermatozoa in our study is consistent with earlier data obtained with a centrifugation force between 300 and 600xg. About 70% to 90% of spermatozoa are recovered depending on temperature, extender, volume, centrifugation time, centrifugai force and volume of supernatant left above pellet (7, 13, 26, 35). Improvement of sperm recovery was observed in Experiments 1 and 3 when centrifugation was performed at 22°C instead of 4°C and when the interval between collection and centrifugation was 10 min instead of 1 h. During centrifugation, the sedimentation rate is influenced by multiple factors. In our study, motility, shape and density of spermatozoa, and density and viscosity of medium may have been different according to the temperature used in each freezing protocol. As density and viscosity increase with decreasing temperature, the temperature could explain by itself the higher recovery of spermatozoa at 22°C. On the other hand, Cochran et al. (7) observed a decreased recovery rate for equine semen centrifuged at 37°C compared to 20°C and suggested that the difference was due to the difference in motility of spermatozoa. Therefore, centrifugation temperature at 22°C could constitute a good compromise between 4°C (high viscosity, high density, low motility) and 37°C (low viscosity, low density, high motility) to recover a maximum number of spermatozoa.

Changing temperature and time of centrifugation/glycerol-addition from 4°C at 1 h after collection to 22°C at 10 min after collection significantly improved post-thaw motility (Experiments 1 and 3) and fertility (Experiment 3). In contrast, Ecot et al. (9) did not observe differences in post-thaw motility when using centrifugation at 4 or 22°C on semen of 11 stallions. In view of the fact that a larger number of stallions was included in our study, it is possible that our results are more representative of the general population of stallions. Fertility per cycle obtained with semen frozen after centrifugation at 22°C for those stallions included in Experiment 3 (56% for 243 cycles) and for stallions not included (51% for 203 cycles) was within the range of the per-cycle fertility obtained with fresh semen (50%, n=ll,598 cycles) or with natural service (58%, n= 14,239) at National Stud in the same period. Severa!hypotheses may explain these results: 1) less exposure to seminal plasma, 2) Jess damage during centrifugation and 3) less damage during hyperosmotic shock due to glycerol.

It bas been suggested that decreased exposure to the combination of egg yolk (4%) and seminal plasma could increase stallion semen motility after storage at 4°C (4, 5, 14). In our laboratory, we recently confirmed this deleterious effect in spermatozoa stored for 7 days at 4°C in INRA82

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extender with 2 or 4% egg yolk added (Magistrini & Nguekam-Feugang, persona! observation). In goats, the interaction between seminal plasma and egg yolk is weil documented. In this species, seminal plasma is deleterious for the survival of spermatozoa chilled or frozen in media containing egg yolk (27). It bas been demonstrated that a bulbourethral gland lecithinase hydrolyzes the egg yolk lecithins resulting in the production of fatty acids and lysolecithin. The lysolecithin causes membrane destabilization (1).

It has been proposed that, during centrifugation, cells are not damaged during sedimentation, but that become injured once they are pelleted (15). These authors suggested that standard conditions often result in a nonhomogenous centrifugai field with substantially lower g-forces at the top than at the bottom. Thus, after 1 h storage (all4/4 protocols), spermatozoa have sedimented spontaneously before centrifugation and may be damaged preferentially during centrifugation. On this basis, one can resuspend spermatozoa before centrifugation to obtain a more homogenous force among spermatozoa. Membrane plasticity could also play a role in survival during centrifugation. Membranes are still in liquid phase at 22°C and could therefore undergo Jess damage during centrifugation at 22°C than at 4°C. In addition, we observed that pellet resuspension is distinctly easier when centrifugation occurred at 22°C than at 4°C. Cochran et al. (7) also observed a more rapid pellet resuspension at 37°C than at 20°C. It is possible that at 4°C, the degree of agglutination after centrifugation is increased, thus accounting for the increased difficulty of cell resuspension. If increased agglutination is present at lower temperatures, it may indicate increased susceptibility to membrane damage (plasmatic or acrosomal) from centrifuging cells with membranes in a more gel than fluid state. Perhaps, the ease of pellet resuspension is progressive between 37 and 4°C and could contribute to the increased survival rate after centrifugation.

Glycerol addition leads to a very rapid shrinkage of the cell because water exits more rapidly than glycerol enters (36). Thus, one could hypothetize that membranes of equine spermatozoa better withstand rapid shrinkage at 22°C than at 4°C (the temperature at which they are in gel phase and perhaps more sensitive to damage). Temperature (8°C to 22°C) raises the permeability coefficient of glycerol in human spermatozoa (11) and can result in different volume variation of the cel!. Addition of glycerol may aid in "plasticizing" the plasma membrane since its presence modified water permeability in murine sperm for temperatures between 4 and 0°C (21). There is evidence that glycerol could alter the membrane bilayer structure (12) and could even be included in the metabolism of human spermatozoa (2). In contrast to other species, physical exchanges between water and glycerol through stallion spermatozoa membrane is poorly documented. Cochran et al. (7) compared the effect of two temperatures for glycerol addition (20°C or 4°C) and observed similar post-thawing motility. Noiles et al. (22), in a preliminary report, observed that stallion spermatozoa are characterized by an extraordinarily high water permeability and a high glycerol permeability at 22°C. Sieme et al. (30) suggested that the deleterious effect of osmotic change on membrane integrity is Jess pronounced at 22°C than at 4°C. Sorne of these observations suggested that glycerol and water could perhaps exchange more rapidly at 22°C than at 4°C in equine spermatozoa.

When using egg yolk in the centrifugation extender, a slow cooling rate appeared detrimental on post-thaw motility in comparison with a moderate cooling rate in Experiment 1 (Slow Cooling 4/4 vs Control 4/4). These results disagree with the higher motility described after a slow cooling rate before storage at 4°C in milk base extender reported by sorne authors (see Introduction). However, these results are in agreement with more recent reports which state that, for stallion or jack spermatozoa, a very slow cooling rate in milk base extender before storage at 4°C is not so critical relative to moderate cooling rate (-0.5°C/min) (4, 5, 10, 29). Another explanation of our results could be that the deleterious effect of seminal plasma and egg yolk (see above) could have occurred during a more active metabolism phase due to a longer stay at higher temperature.

In Experiment 2, after centrifugation at 22°C, freezing directly from 22°C to -196°C resulted in a lower motility compared to cooling to 4°C in 1 h before freezing. This is in agreement with results

reported previously in INRA82 (13). Possibly this cooling rate is incorrect for this cell type, particularly with the relatively low concentration of cryoprotectant, compared to that used with other cell types.

At the French National Stud freezing centers, the Control 4/4 protocol resulted in a selection rate of ejaculates (ejaculates selected based on post-thaw motility more than 35%) of 0%, 1-33%, 33-66% and more than 66% in 19%, 30%, 27% and 24% of the stallions, respectively (34). In a recent survey of 31 young staJJions, the Delayed Cooling 22/4 protocol resulted in a selection rate of 13%, 0%, 13% and 74%, respectively (unpublished data).

The results of this study demonstrate that centrifugationlglycerol-addition at 22°C, 10 min after collection, significantly improves the recovery rate of spermatozoa after centrifugation, post-thaw motility and fertility resulting in a per-cycle fertility in the same range as observed with fresh semen.

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