

Effect on post-thaw viability of equine sperm using stepwise addition of dimethyl formamide and varying cooling and freezing procedures

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Abstract

The aim of this study was to verify the effect on equine sperm viability post-thawing by adding the cryoprotectant dimethyl formamide (5%) at 22°C, using a single step or by adding one tenth of the cryoprotectant at 1-min intervals into the extender, with or without slow cooling to 5°C before freezing in liquid nitrogen vapor. INRA 82 was used as the semen extender. Post-thawing sperm cells showed increased viability parameters ($P < 0.05$) when the cryoprotectant was added in one-tenth fractions, at 1-min intervals and using slow cooling to 5°C before freezing ($P < 0.05$). A second aim was to verify whether increasing the time interval between the addition of each tenth part of dimethyl formamide, cooling the samples to 5°C, increasing equilibration time and using different freezing rates would improve post-thawing sperm viability. Sperm viability analyses in this part of the study showed no significant improvement in sperm parameters in any of the treatments ($P > 0.05$). It may be concluded that the stepwise addition of dimethyl formamide at 1-min intervals to the INRA 82 extender and using slow cooling to 5°C before freezing, independent of the freezing system, preserved equine sperm viability post-thawing more than any of the methods tested.

Keywords: dimethyl formamide, equine, frozen semen.

Introduction

Equine sperm freezing procedures are still inadequate considering the extended variation found in results between individuals and even between ejaculates of the same individual. Factors affecting the freezing process can be the type of permeable cryoprotectant used, the cooling rate, the composition of the extender, resistance of the sperm cell to osmotic stress, among others, and the possible interaction between factors (Aman and Pickett, 1987; Squires *et al.*, 1999). Several approaches have been tested during the last decades with the aim of improving equine sperm freezing procedures. The search for alternative cryoprotectants has been one of them. Glycerol has been extensively used as cryoprotectant (Cochram *et al.*, 1984; Squires *et al.*, 1999; Vidament *et al.*, 2002), but ethylene glycol (Barry and Anthony, 2001; Cottorello, 2002; Snoeck, 2003), propanodiol (Ashwood-

Smith, 1987; Keith, 1998; Barry and Anthony, 2001), among others, have been tested.

Cryoprotectant agents are required to enhance cell survival after cooling and/or warming, however, their presence could potentially induce damaging volume expansion and contraction (Gilmore *et al.*, 1997). Osmotic stress may provoke cell injury during freezing and thawing (Mazur, 1984; Watson, 1996; Leibo and Bradley, 1999; Meyers, 2005). It is also recognized that the cryoprotective capacity of any cryoprotectant agent varies widely across cell and tissue type, therefore, efficacy of each cryoprotectant agent must be studied for each cell type under investigation (Karow, 1969). Consequently, optimizing the procedures for adding cryoprotectant agents before cooling and removal and after thawing is required to improve cryopreserved cell viability according to specific cell characteristics (Gilmore *et al.*, 1997). For human sperm, it has been shown that due to cryoprotectant addition, the volume expansion and contraction upper limit is 1.1 times of isotonic volume and the lower limit is 0.75 times isotonic volume if 5% motility loss is chosen as a criterion (Gao *et al.*, 1995).

Rapid addition and, more importantly, rapid removal of glycerol from equine unfrozen spermatozoa resulted in a marked decrease in motility and viability (Barry and Anthony, 2001). Of the four cryoprotectant agents tested by the previous authors, ethylene glycol caused least osmotic damage showing that equine spermatozoa had a limited osmotic tolerance to anisotonic conditions.

Recently, dimethyl formamide has been considered an alternative cryoprotectant to prevent equine spermatozoa damage (Graham, 2000; Vidament *et al.*, 2002; Moffet *et al.*, 2003), particularly in cases of spermatozoa from stallions sensitive to the freezing process (Alvarenga *et al.*, 2005). Since then, the concentration of 5% dimethyl formamide has been indicated for use in extenders for equine spermatozoa (Medeiros *et al.*, 2002).

Considering that there are few studies concerning the osmotic tolerance of equine spermatozoa and based on the above premises, this study aimed to verify sperm viability post-thawing, testing a single step or stepwise addition of dimethyl formamide at room temperature, as well as to verify an eventual interaction of the addition form with equilibration time at 5°C. Based on the results of the first experiment, a second

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one was carried out aiming to test whether increasing the time length intervals between stepwise cryoprotectant insertion might be a factor which could enhance sperm viability post-thawing and if stepwise addition would interact with equilibration time length and cooling rate. The hypothesis was that post-thaw sperm viability evaluated *in vitro* in anisotonic condition would improve using stepwise addition of dimethyl formamide before the cooling phase and an additional improvement could be reached adjusting the equilibration time or cooling rate.

Materials and Methods

Experiment 1

Nine stallions, ranging in age from 4 to 15 years (Mangalarga Marchador breed), were used. Sperm samples were collected daily by artificial vaginal (Hannoverian model) until stabilization of the extragonadal sperm reserves. Thereafter, semen collections were maintained at 3 day intervals. One ejaculate per stallion was submitted to the freezing process when the following conditions were reached: $\geq 60\%$ progressive motility, $\leq 30\%$ of total sperm morphology abnormality and at least 3 billion sperm cells per ejaculate.

INRA 82 extender with 2% egg yolk (Palmer, 1984) was used to process the semen. The INRA 82 was chosen as the standard extender due to its known efficacy and the fact that sperm cells could easily be identified, thus, making motility evaluation feasible under light microscopy.

Dimethyl formamide (5%) was used as cryoprotectant. The osmolarity of the extender with and without the cryoprotectant was 1063 mOsmol/L and 317.5 mOsmol/L, respectively.

Gel free semen was diluted 1:1 in the extender free of cryoprotectant and centrifuged at 600 g for 12 min. Sperm cells were resuspended, and the sperm concentration was evaluated using a Neubauer chamber. After centrifugation, extender free of cryoprotectant was used up to completion of half of the total volume necessary to obtain 100×10^6 spermatozoa per ml (initial dilution). Final volume was reached using the extender with 10% dimethyl formamide to obtain a final 5% cryoprotectant concentration. The extender with the cryoprotectant was added at room temperature ($\sim 25^\circ\text{C}$) either in a single step, 10 min after an equilibration time at 37°C , or by adding in 10 steps of fixed volume at 1-min intervals starting immediately after initial dilution. The samples were then loaded in 0.5 ml straws.

Treatments tested were: 1) addition of the cryoprotectant in a single step followed by immediate freezing in nitrogen vapor (4 cm above the nitrogen level for 15 min; Tr1); 2) addition of the cryoprotectant in 10 steps of fixed volume followed by immediate

freezing in nitrogen vapor (Tr2); 3) addition of the cryoprotectant in a single step followed by cooling at $-0.25^\circ\text{C}/\text{min}$ down to 5°C using a programmable freezer (TK 3000® Tetakon – Nutricell) and then freezing in nitrogen vapor (Tr3); 4) addition of the cryoprotectant in 10 steps of fixed volume followed by cooling at $-0.25^\circ\text{C}/\text{min}$ down to 5°C using programmable freezer (TK 3000® Tetakon – Nutricell) and then freezing in nitrogen vapor (Tr4).

Experiment 2

Another set of six stallions, ranging in age from 4 to 8 years (Mangalarga Marchador breed), were used in Experiment 2. Stallion preparation and seminal quality parameters for ejaculate selection were the same as described in Experiment 1. One ejaculate was collected per stallion and semen manipulation and extender used for the freezing process were the same as in Experiment 1. Treatments tested were: 1) addition of the cryoprotectant in 10 steps of fixed volume at 1-min intervals; 2) addition of the cryoprotectant in 10 steps of fixed volume at 2-min intervals; 3) addition of the cryoprotectant in 10 steps of fixed volume at 3-min intervals. Samples of the three additional cryoprotectant treatments were cooled from room temperature down to 5°C using a programmable freezer (TK 3000® Tetakon – Nutricell) at a rate of $-0.25^\circ\text{C}/\text{min}$. When 5°C was reached, procedures were as follows: 1) immediate freezing at 4 cm above the liquid nitrogen level for 15 min; 2) additional equilibration time at 5°C of 45 min followed by freezing in nitrogen vapor (as described above); 3) additional equilibration time at 5°C of 45 min and using a programmable freezer at a rate of $-10^\circ\text{C}/\text{min}$ until -127°C . For both experiments, after freezing all samples were plunged and stored in liquid nitrogen until post-thawing evaluation. Thawing was done at 52°C for 7 s, followed by immersion of the samples in a water bath at 37°C for 30 s.

Sperm evaluation methods

Total and progressive sperm motility (%) and speed (scale 1-5) were evaluated by three qualified technicians unaware of the origin of the sample in analysis, using light microscopy (400X). The mean value obtained by the three technicians was used for analysis. Total and progressive sperm motility and speed were assessed immediately post-collection and post-thawing and progressive motility was continued to be evaluated at 30 min intervals under incubation at 37°C .

The functional integrity of the sperm plasma membrane was evaluated pre-freezing and 30 min post-thawing by the hypoosmotic test (HOST). One hundred cells per sample were analyzed by phase contrast microscopy (400X) and the percentage of cells reactive to HOST was calculated as follows: $\text{HOST} (\%) = (\% \text{ of})$

alterations in the tail region after HOST) – (% of alterations in the tail region before HOST; Melo and Henry, 1999)

Sperm morphology was evaluated in samples preserved in saline formaldehyde buffer by contrast phase microscopy (Olympus CBA Microscopy; 1000X). The structural integrity of the sperm plasma and acrosomal membranes was evaluated by fluorescent dyes: propidium iodide (IP) and carboxyfluorescein diacetate (CFDA) (Harrison and Vickers, 1990), modified by Zuccari (1998). Sperm cells were classified in three categories: intact, semi-damaged (acrosomal membranes still intact), and damaged.

Statistical analyses

A random experimental design was used for Experiments 1 and 2. The effects of the form of cryoprotectant addition and cooling were assessed by the analysis of variance and the means obtained for the different parameters (total motility, progressive motility, functional sperm plasma membrane integrity, and structural integrity of the plasma and acrosomal

membranes) were evaluated by the Duncan test at 5% of significance. In Experiment 2, the quantitative data (hyposmotic swelling test, structural integrity of the plasmatic membranes and integrity of the acrosomal membrane) were analyzed to verify the normality (Lilliefors test) and homogeneity of variance (Cochran and Bartlett's test). Means were compared ($P < 0.05$) by the Duncan's test. The qualitative data (total and progressive motility, speed and thermal-resistance test) were analyzed by the non-parametric test according to Kruskal-Wallis. Correlations between the variables were carried out according to Spearman. The program SAEG-8.0 version was used for analysis.

Results

Experiment 1

Sperm motility, speed and percentage of spermatozoa reactive to the hyposmotic test evaluated pre-freezing after cryoprotectant addition and post-thawing and the percentage of intact sperm evaluated by fluorescent dyes immediately post-thawing, are shown in Table 1.

Table 1. Equine sperm characteristics pre-freezing after cryoprotectant addition and post-thawing.

	Total Motility (%)	Progressive Motility (%)	Speed (1-5)	Intact (%)	HO T1 (%)	HO T30 (%)
Pre-freezing cryoprotectant addition						
Single step	80.7 ± 6.0	72.9 ± 7.0	3.4 ± 0.3		55.3 ± 15.4	
Stepwise addition	77.1 ± 17.8	70.0 ± 16.2	3.3 ± 0.8		52.0 ± 16.6	
Post-thawing						
Tr1	42.3 ± 14.0 ^{aA}	35.8 ± 12.9 ^{aA}	2.7 ± 0.4 ^{aA}	35.6 ± 14.4 ^{aA}		10.7 ± 11.4 ^{aA}
Tr2	52.5 ± 18.4 ^{bA}	46.5 ± 18.1 ^{bA}	3.0 ± 0.6 ^{aA}	37.8 ± 11.2 ^{aA}		14.7 ± 9.8 ^{bA}
Tr3	55.4 ± 8.0 ^{aB}	49.3 ± 7.8 ^{aB}	2.9 ± 0.2 ^{aA}	36.0 ± 8.6 ^{aA}		21.0 ± 7.1 ^{aB}
Tr4	63.6 ± 14.3 ^{bB}	58.2 ± 14.0 ^{bB}	2.9 ± 0.8 ^{aA}	45.2 ± 13.6 ^{aA}		27.1 ± 10.7 ^{bB}

Intact = spermatozoa with plasma and acrosomal membranes integrity by the fluorescence test; HO = spermatozoa reactive to the hyposmotic test pre-freezing after final dilution (T1) and 30 min post-thawing (T30; %); Tr1 = single step cryoprotectant addition; Tr2 = addition of the cryoprotectant in 10 steps of fixed volume at 1-min intervals, both treatments without slow cooling before freezing in the nitrogen vapor; Tr3 = single step cryoprotectant addition; Tr4 = Addition of the cryoprotectant in 10 steps of fixed volume at 1-min intervals. Slow cooling (0.25°C/min) from room temperature down to 5°C before freezing in nitrogen vapor was used for the last two treatments. For each sperm characteristic, different low case letters considering Tr1 x Tr2 and Tr3 x Tr4 indicate $P < 0.05$; different upper case letters considering Tr1 x Tr3 and Tr2 x Tr4, indicate $P < 0.05$. Average of nine stallions.

The form of cryoprotectant addition did not alter sperm parameters evaluated post-final dilution. Immediately post-thawing, total and progressive motility were higher when the cryoprotectant was added stepwise for each of the two cooling systems ($P < 0.05$) and total and progressive motility were higher ($P < 0.05$) when using slow cooling before freezing for both ways of cryoprotectant addition. No interaction was observed between forms of cryoprotectant addition and presence

or absence of slow cooling before freezing.

The values for progressive motility post-thawing at 30 and 60 min of incubation at 37°C were higher in the treatments where the cryoprotectant was added stepwise, independently of slow cooling or not before freezing ($P = 0.06$), and in the treatments where the spermatozoa were cooled slowly before freezing, independently of the form of cryoprotectant addition ($P < 0.05$).



The correlation coefficients found in Experiment 1 were 0.49 ($P = 0.001$), 0.36 ($P = 0.02$) and 0.32 ($P = 0.03$) between, respectively, progressive motility and the HOST, progressive motility and integrity of the sperm membranes and between integrity of the sperm membranes and reactivity to the HOST ($P = 0.02$).

Experiment 2

Table 2 shows the results for sperm motility

and speed and reactivity to the hypoosmotic test evaluated before freezing and after thawing and percentage of sperm with intact membranes evaluated by fluorescent dyes immediately after thawing. Post-thawing, there were no differences between treatments on all evaluated parameters ($P > 0.05$).

No significant difference ($P > 0.05$) was observed at any observation time on progressive sperm motility between treatments during the incubation at 37°C after thawing.

Table 2. Equine sperm characteristics pre-freezing and post-thawing.

	Total Motility (%)	Progressive Motility (%)	Speed (1-5)	Intact (%)	HO T1 (%)	HO T30 (%)
Pre-freezing	73.3 ± 3.7	68.3 ± 3.7	3.6 ± 0.2		56.2 ± 8.6	
Post-thawing						
Curve 1						
T10	47.5 ± 7.5	40.8 ± 6.4	3.1 ± 0.2	47.7 ± 10.5	--	36.8 ± 4.2
T20	44.2 ± 7.6	38.3 ± 8.8	3.1 ± 0.2	45.6 ± 7.4	--	38.7 ± 7.0
T30	40.8 ± 5.8	35.8 ± 5.8	3.1 ± 0.2	44.3 ± 12.2	--	37.0 ± 4.0
Curve 2						
T10	42.5 ± 7.6	37.5 ± 7.6	3.1 ± 0.2	48.1 ± 5.4	--	39.2 ± 5.5
T20	39.2 ± 3.8	34.2 ± 3.8	2.8 ± 0.4	46.7 ± 10.9	--	38.5 ± 5.8
T30	41.7 ± 7.5	36.7 ± 7.5	2.7 ± 0.9	50.5 ± 4.1	--	34.2 ± 5.9
Curve 3						
T10	39.2 ± 3.8	33.3 ± 4.1	2.9 ± 0.2	46.1 ± 10.2	--	38.5 ± 4.8
T20	42.5 ± 7.6	37.5 ± 7.6	3.1 ± 0.2	47.3 ± 5.5	--	40.7 ± 6.8
T30	42.5 ± 5.2	36.7 ± 6.1	3.1 ± 0.2	46.6 ± 12.6	--	39.2 ± 4.7

Intact = spermatozoa with plasma and acrosomal membrane integrity by the fluorescence test (%); HO = spermatozoa reactive to the hypoosmotic test pre-freezing after final dilution (T1) and 30 min post-thawing (T30; %); Curve 1: cooling to 5°C (-0.25°C/min) without additional equilibration time at 5°C and freezing in nitrogen vapor (4 cm above the nitrogen level); Curve 2: adding 45 min of equilibration time after reaching 5°C followed by freezing in nitrogen vapor. Curve 3: adding 45 min of equilibration time after reaching 5°C followed by freezing in a programmable freezer at a rate of -10°C/min, until -127°C. T10: addition of the cryoprotectant in 10 steps of fixed volume at 1-min intervals; T20: addition of the cryoprotectant in 10 steps of fixed volume at 2-min intervals; T30: addition of the cryoprotectant in 10 steps of fixed volume at 3-min intervals. Average of six stallions.

Positive correlations of low magnitude were observed between the percentage of spermatozoa reactive to the hypoosmotic test and total and progressive motility ($R = 0.46$; $P = 0.0005$). Negative correlations of low magnitude were found between the percentage of spermatozoa with intact plasma and acrosomal membranes and total and progressive motility ($R = -0.10$; $P = 0.46$) and speed as well as between the reactivity to the hypoosmotic test and the percentage of spermatozoa with intact plasma and acrosomal membranes ($R = -0.21$; $P = 0.13$).

Discussion

This study focuses on the effect of addition of the cryoprotectant because technically it is easier to handle in field conditions than to cope with the

cryoagent removal just prior to the insemination process. It has been shown that 2% dimethyl formamide (v/v) provided better post-thaw motility than 1%, 3%, and 5% when “good freezer” stallions were used (Vidament *et al.*, 2002). In contrast, Gomes *et al.* (2002), Medeiros *et al.* (2002), and Squires *et al.* (2004) showed that greater concentration of dimethyl formamide (5% v/v or 0.6 and 0.9 M) resulted in enhanced post-thawing motility, particularly with so called “bad freezer” stallions (Alvarenga *et al.*, 2003). As the stallion breed used in this study are considered “bad freezers”, 5% dimethyl formamide was used.

The effect of the first contact of dimethyl formamide on the sperm cells was evaluated before freezing. Exposing sperm cells to permeating cryoprotectant sperm cells will expose them to an osmotic shock. Cells will first shrink as water leaves

through the plasma membrane, and then swell as water re-enters together with the cryoagent. The degree of volume expansion and contraction caused by the osmotic shock may be detrimental to the sperm cell rather than the nature of the solute (Gao *et al.*, 1997). In the present study, directly after cryoprotectant addition in a single step or when added stepwise, progressive sperm motilities and speed or reaction to the hypoosmotic test did not change (Table 1). By that time, if any changes occurred in the sperm cells, it could not be detected by the proposed methodology.

When spermatozoa were analyzed immediately post-thawing, a significantly greater decrease ($P < 0.05$) of total and progressive motility as well as the percentage of cells reactive to the hypoosmotic test was observed in the treatments where the cryoprotectant was added in a single step compared to the values obtained when dimethyl formamide was added stepwise. This was noticed in both treatments: with or without slow cooling down to 5°C before freezing (Table 1) and also up to 60 min of incubation time after thawing. Nevertheless, adding the cryoprotectant in a single step or stepwise did not affect the structural integrity of the sperm membranes evaluated by the fluorescence test.

All these findings combined suggest that single step addition affected more the sperm mechanisms than the cell structure itself, including motility and the capacity of the membrane to allow the entrance and retention of water in the tail region when submitted to an hypoosmotic solution. It is known that membrane composition varies according to the location in the sperm cells (Watson, 1995; Meyers, 2005) and is structurally different in each sperm segment. As in both treatments where one step cryoprotectant addition was used, deleterious effects occurred in the tail, which may indicate that this sperm segment is more vulnerable to the transient hyperosmotic shock. However, as in the sperm head, only the structure of the membranes was evaluated, and no effect was detected. Therefore, it cannot be excluded that other changes could have been missed.

Barry and Anthony (2001) demonstrated that the osmotic shock caused by rapid addition and removal of glycerol, ethylene glycol, dimethylsulfoxide or propylene glycol in unfrozen equine sperm resulted in a remarkable decline of motility, viability, and potential of the mitochondrial membrane. This effect was more pronounced with glycerol and less with ethylene glycol, indicating that each cryoagent causes different osmotic stress on equine sperm cells. Dimethyl formamide has a lower molecular weight (72) than glycerol (93) (Moffet *et al.*, 2003), which potentially results in a greater ease to bypass the sperm membranes than glycerol. This may result in a faster equilibration time between the intra- and extra-cellular regions resulting, theoretically, in a lower potential to cause damage to the sperm cell through osmotic shock. However, despite this characteristic of dimethyl formamide, the results

indicated a decrease in the sperm viability post-thawing when it was added in a single step. In an experiment carried out with human spermatozoa, it was clear that during the addition of 1M of glycerol in a single step, the change of the cell volume exceeded the limit tolerable for sperm cells. Conversely, the stepwise addition and removal of the cryoprotectant reduced stress to tolerable limits (Gao *et al.*, 1995).

In the present experiment, no interaction was observed between the form of cryoprotectant addition and use or not of slow cooling before freezing. It was observed that slow cooling of the diluted semen down to 5°C before freezing preserved better progressive motility and reactivity of the sperm cells to the hypoosmotic test independently of the cryoagent addition manner (Table 1) and this effect also lasted up to 60 min of post-thawing incubation time. It has been shown that slow cooling at a moderate rate before freezing in extenders containing skim milk and 2% egg yolk resulted in a significant increase in post-thawing motility (Vidament *et al.*, 2000; Knop *et al.*, 2005). The fact that stepwise addition and slow cooling before freezing has been shown to be the more promising process and that slow cooling was also beneficial when adding cryoagent in one step, suggests that a longer permanence of the sperm cells in liquid phase before freezing allowed them to recover from the shock caused by the addition of the cryoprotectant and gave them time to better interact with the extender.

The second experiment was designed to use the best treatment of Experiment one as control, that is, cryoagent added step wise and slow cooling before freezing, and to test whether increasing the time interval between addition steps of fixed volumes of cryoagent in association with increasing the equilibrium time at 5°C and adding a slower freezing curve to the process, might result in additional improvements in post-thawing sperm viability.

The results presented in Table 2 showed that changing the time interval between steps of addition of fixed cryoagent volume and increasing the equilibration time at 5°C did not bring any further benefit to the one observed in Experiment 1. Sperm motility, reaction to the hypoosmotic test, and membrane integrity evaluated by fluorescent probes were equal in all treatments. The same was observed for progressive motility during the incubation time post-thawing.

The time needed for glycerol sperm penetration is 3 to 4 min (Berndtson and Foote, 1972). Considering the lower molecular weight of dimethyl formamide as well as the temperature it was added (room temperature), the 10 min given between addition steps of dimethyl formamide in the control treatment of Experiment 2 may have already surpassed the time needed for cryoagent penetration and equilibration between intra and extra compartments. Instead of increasing the time between fixed volume additions, it might have been more adequate to further decrease the



amount of cryoagent added per step.

Increasing the equilibration time at 5°C before freezing did not bring any further advantage and the two freezing processes used (nitrogen vapor or the programmable freezer with the cooling rate here tested) showed to work evenly.

It may be concluded that the stepwise addition of dimethyl formamide in the INRA 82 extender associated to a slow cooling down to 5°C followed by immediate freezing, showed a beneficial effect on sperm survival post-thawing. Of all time intervals tested between additions of fixed volume of cryoagent, 10 min showed the best results.

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