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Viability and fertility of stallion semen frozen with ethylene glycol and acetamide as a cryogenic agent

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Abstract

This study was designed to determine the effect of cooling at a rate of -0.13°C/min or any slow cooling prior to freezing on stallion sperm and to determine the effect of two thawing temperatures on the viability of sperm frozen using a lactose-EDTA-egg yolk extender (LAC) with 3.5% ethylene glycol (LAC 3.5% EG) or a LAC with the additional incorporation of methyl cellulose, trehalose and acetamide (LAC+5% AC). No differences were observed in the post-thaw parameters of sperm frozen using either one of the cryogenic agents or frozen with or without previous slow cooling. Thawing at 75°C was better than at 37°C ($P < 0.05$). An interaction was observed between the cryoprotectant and the freezing protocol ($P < 0.05$). A conception rate of 23.3% was obtained after AI using equine semen frozen with LAC+5% AC.

Keywords: cryopreservation, equine, methyl cellulose and trehalose, semen.

Introduction

The preservation of post-thaw stallion sperm motility and fertility is variable among stallions (Vidament *et al*., 1997; Alvarenga *et al*., 2005; Vidament, 2005), particularly for certain breeds. This variability indicates that the techniques currently used may not be suitable for some stallions (Alvarenga *et al*., 2005). The use of glycerol as a cryoprotectant could be one factor responsible for this variation. Even when using adequate glycerol concentrations, detrimental effects on sperm, including loss of motility and integrity of the membranes, are not avoided (Pace and Sullivan, 1975; Moffet *et al*., 2003; Vidament, 2005). These results encouraged the testing of several other cryoprotectants for equine sperm, including ethylene glycol and certain amides (Alvarenga *et al*., 2000; Keith *et al*., 2000; Henry *et al*., 2002; Medeiros *et al*., 2002; Squires *et al*., 2004; Alvarenga *et al*., 2005), together with egg yolk and/or skim milk or low-density lipoprotein from egg yolk plasma (Pillet *et al*., 2011) and large sugar molecules (Squires *et al*., 2004; Snoeck *et al*., 2007; Terraciano *et al*., 2008; Fagundes *et al*., 2010). Neves Neto *et al*. (1995) have demonstrated good pregnancy rates with semen frozen using ethylene glycol. Other works showed that amides are useful for cryopreserving sperm from many species without detrimental effects on fertility, indicating that amides could be potentially useful alternatives for the cryopreservation of equine sperm.

The objectives of the following experiment were to evaluate the effectiveness of acetamide with methyl cellulose and trehalose and ethylene glycol as cryoprotectants for freezing equine sperm with or without previous slow cooling and to test two thawing temperatures. The fertility of the semen that was frozen using acetamide was also investigated.

Materials and Methods

A 2 x 2 x 2 factorial experiment was designed to determine which of two cryopreservation extenders, freezing rates and thawing temperatures were the most optimal in the preservation of stallion spermatozoa. Single ejaculates from 12 stallions of the Mangalarga Marchador, Piquira or Poney breeds were collected. The stallions were selected for their ability to produce ejaculates with more than 100 x 10^6 sperm/ml and ≥70% progressive sperm motility after a week of daily semen collection to stabilize the sperm reserve. Immediately after collection, semen free of gel was diluted 1:1 (v:v) in an EDTA–glucose extender (Martin *et al*., 1979) and centrifuged at 400 x g for 10 min. The supernatant was discarded, and the pellets were resuspended to a final sperm concentration of 100 x 10^6/ml. In order to avoid inaccuracy during sperm motility evaluation, the egg yolk of the extender was previously homogenized. The lactose-EDTA-egg yolk (LAC) extender (Martin *et al*., 1979) was used with the following cryoprotectants in place of glycerol: 3.5% ethylene glycol (LAC 3.5% EG; 1130 mOsmol/l - pH 6.0) and 5% acetamide with the incorporation of 0.5% methyl cellulose and 0.165% trehalose (LAC+ 5% AC; 1480 mOsmol/l – pH 6.4). All chemicals and reagents were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. The osmotic pressure and pH of the LAC extender before the addition of the cryoprotectants were 356 mOsmol/l and 6.5, respectively.

The extenders were selected based on previous results (Henry *et al*., 2002). The first extender was

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chosen because it was evenly effective among stallions compared to other extenders but contained low percentage of cryogenic agent (3.5% ethylene glycol), and the second extender (LAC+5% AC) was selected because it preserved the most post-thaw sperm parameters compared to other LAC+AC extenders tested while preserving the structural integrity of the sperm membranes. In the former experiment, glycerol was used as the control group and showed no notable improvement in sperm viability compared to ethylene glycol and acetamide.

The samples diluted in both extenders were either cooled from room temperature to 5°C using an average cooling rate of -0.13°C/min and frozen or were directly frozen in 0.5 ml straws by placing them 3 cm above the liquid nitrogen level for 10 min. The samples were thawed at 37°C for 30 s or at 75°C for 7 s followed by immersion in a water bath at 37°C for 5 s.

Progressive sperm motility and sperm membrane integrity were evaluated immediately after thawing. Sperm motility was evaluated by two experienced observers using a light microscope (Olympus® CX 31) at 400X magnification. The samples were laid on a pre-warmed (37ºC) slide and covered with a cover slide. The structural integrity of the plasma and acrosomal membranes was evaluated using a fluorescent microscope (400X; Olympus® CX 51) after staining the sperm with the fluorescent dyes carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) according to the method of Harrison and Vickers (1990). Staining with CFDA was assessed using the standard fluorescein filter set, while staining with PI was assessed using the standard rhodamine filter set. The functional integrity of the plasma membrane was assessed using the hypoosmotic swelling test (HOST) with 100 µl of the sample diluted in 1.0 ml of a 100 mOsmol/l sucrose solution. The diluted samples were first incubated in a water bath at 37ºC for 30 min and were subsequently fixed with 500 µl of buffered formalin-saline, and 100 cells were evaluated using a phase contrast microscope (1000x; Olympus® BX 41). The percentage of cells reactive to HOST was calculated according to the method of Melo and Henry (1999).

The ejaculates from a fertile Mangalarga Marchador stallion were frozen in LAC+5% AC to test the fertility rate. The semen was processed as described above. The extended semen was submitted to slow cooling to 5ºC (-0.13°C/min) and frozen using a static liquid nitrogen vapor prior to submersion into liquid nitrogen (-196°C). The straws were thawed at 75°C for 7 s followed by immersion in a 37°C water bath for 5 s.

Twenty-one cycling mares, 8 to 20 yr old, of unknown fertility were used. Insemination was performed during 30 ovulatory estrous cycles, 27 of which used conventional post-cervical seminal deposition and 3 of which used deposition during ovulatory estrous in the horn tip *ipsis* lateral to the side of the ovulation occurrence, as determined by the hysteroscopic method (Morris *et al*., 2000). One insemination per estrous was performed after the occurrence of ovulation (maximum 6 h post-ovulation) using an average of 200 x 10^6 progressively motile sperm diluted in 3.6 ± 0.8 ml (conventional method) and an average of 30 x 10^6 progressively motile sperm in 1.0 ml doses (hysteroscopic method). The average post-thaw motility was 39.7 ± 7.8%. The average interval between thawing and AI was 10.8 ± 4.0 min for the conventional method and between 15 to 30 min for the hysteroscopic method. Pregnancy was diagnosed by ultrasound between 14 and 16 days after AI.

The Statistical Package for Social Sciences (SPSS, v 11.0, Chicago, USA) software was used for the statistical analysis. All percentage data were transformed prior to analysis using arcsine (arcsin √X/100 transformation). The results express the average of the evaluation of three straws per treatment. Statistical analysis was performed using ANOVA. Data from the experiment were analyzed using Student's t-test. Differences of $P < 0.05$ were considered to be statistically significant.

Results

The overall post-thaw sperm progressive motility was 39.1 and 42.3% when the sperm were extended in LAC 3.5% EG or LAC+5% AC, respectively ($P > 0.05$), regardless of the cooling curve or thawing temperatures, 40.9 and 40.4% when slow cooling was performed before freezing or when the sperm were directly frozen after final dilution, respectively ($P > 0.05$), regardless of the cryogenic agent or thawing temperature and 43.4 and 38.0% when the thawing temperatures were 75 or 37ºC, respectively ($P < 0.05$), regardless of the cryogenic agent or cooling curve. For the LAC 3.5% EG extender, sperm motility was not preserved as effectively ($P < 0.05$) than with the LAC+5% AC extender when using slow cooling prior to freezing. No other differences were found among the treatments. Thawing at 75ºC always provided high sperm motility percentages, regardless of the extender or cooling curve employed (Table 1).

The overall percentage of sperm reacting to the hypoosmotic test were 28 and 27.8% when using LAC 3.5% EG or LAC+5% AC extenders, respectively ($P > 0.05$), and 28.8 and 27.0% when using slow cooling prior to freezing or freezing directly after the final dilution, respectively ($P > 0.05$) and 30 and 25.8% when the sperm were thawed at 75 or 37ºC, respectively ($P > 0.05$). The sperm reacted similarly to the hypoosmotic test, regardless of the extender, cooling curve or thawing temperature, but there was a trend towards higher reactivity to the hypoosmotic test when the samples were thawed at 75ºC (Table 2).

Table 1. Effect of combination of two extenders, two freezing rates and two thawing temperatures on post-thaw stallion sperm progressive motility.

Extender	Freezing method	Thawing 37°C / 30s	Temperature 75°C / 7s
LAC 3.5% EG	With cooling rate	35.2 ± 11.2^{bB}	37.1 ± 12.8^{aB}
	Without cooling rate	38.1 ± 12.3^{bAB}	45.8 ± 14.4^{aAB}
LAC+5% AC	With cooling rate	42.5 ± 12.7^{bA}	49.0 ± 9.1^{aA}
	Without cooling rate	37.1 ± 14.0^{bAB}	41.7 ± 13.7^{aAB}

LAC 3.5% EG: lactose-EDTA-egg yolk extender with 3.5% ethylene glycol; LAC+5% AC: lactose-EDTA-egg yolk + methyl cellulose + trehalose and 5% acetamide. Slow cooling: cooling at an average rate of -0.13ºC/min from room temperature to 5°C. ^{ab}Means within a line with no common superscript letter differed ($P < 0.05$). ^{AB}Means within a column with no common superscript letter differed ($P < 0.05$).

Table 2. Effect of combination of two extenders, two freezing rates and two thawing temperatures on post-thaw stallion sperm reacting to the hypoosmotic test.

Extender	Freezing method	Thawing 37°C / 30s	Temperature 75°C / 7s
LAC 3.5% EG	With cooling rate	25.7 ± 19.3	27.2 ± 18.4
	Without cooling rate	29.9 ± 16.3	29.3 ± 15.6
LAC+5% AC	With cooling rate	27.6 ± 19.6	34.8 ± 14.8
	Without cooling rate	20.2 ± 9.6	28.8 ± 17.2

LAC 3.5% EG: lactose-EDTA-egg yolk extender with 3.5% ethylene glycol; LAC+5% AC: lactose-EDTA-egg yolk + methyl cellulose + trehalose and 5% acetamide. Slow cooling: cooling at an average rate of -0.13ºC/min from room temperature to 5°C.

The overall frequency of sperm with intact plasma and acrosomal membranes after thawing was 39.5 and 37.1% for LAC 3.5% EG and LAC+5% AC extenders, respectively ($P > 0.05$), 40.9 and 35.8% for the sperm slowly cooled to 5ºC before freezing or directly frozen after final dilution, respectively ($P > 0.05$) and 42.5 and 34.1% when the sperm were thawed at 75 or 37ºC, respectively ($P < 0.05$). The integrity of the plasma and acrosomal membranes was less preserved ($P < 0.05$) when the sperm were extended in LAC+5% AC and frozen without previous slow cooling than when the sperm were extended in LAC+5% AC and frozen with previous slow cooling or when extended in LAC 3.5% EG and frozen without slow cooling. It was also evident that independently of the extender and cooling rate, the membranes were better preserved when the thawing temperature was 75ºC ($P < 0.05$). There was no further difference among the treatments regarding the integrity of the sperm membranes (Table 3).

The fertility rate of the sperm frozen in LAC+5% AC was 22.2% (6/27 ovulatory estrous cycles) for mares inseminated by the conventional method and 33.3% (1/3 ovulatory estrous cycle) using the hysteroscopic method. The overall pregnancy rate was 23.3% (7/30 ovulatory estrous cycles).

Table 3. Effect of combination of two extenders, two freezing rates and two thawing temperatures on post-thaw stallion percentage of plasma and acrosomal intact sperm membranes.

Extender	Freezing method	Thawing 37°C / 30s	Temperature 75°C / 7s
LAC 3.5% EG	With cooling rate	35.1 ± 15.3^{bAB}	43.2 ± 17.0^{aAB}
	Without cooling rate	37.3 ± 11.6^{bA}	42.4 ± 15.6^{aA}
LAC+5% AC	With cooling rate	36.9 ± 12.1^{bA}	48.3 ± 12.8^{aA}
	Without cooling rate	27.7 ± 12.1^{bB}	36.3 ± 11.7^{aB}

LAC 3.5% EG: lactose-EDTA-egg yolk extender with 3.5% ethylene glycol; LAC+5% AC: lactose-EDTA-egg yolk + methyl cellulose + trehalose and 5% acetamide. Slow cooling: cooling at an average rate of -0.13 ºC/ min from room temperature to 5°C. ^{ab}Means within a line with no common superscript letter differed ($P < 0.05$). ^{AB}Means within a column with no common superscript letter differed ($P < 0.05$).

Discussion

The overall post-thaw sperm motility and the structural and functional integrity of the membranes, regardless of the cooling curves and thawing temperatures, were evenly preserved using either LAC with 3.5% EG or LAC with 5% AC with methyl cellulose and trehalose, showing the same trend reported previously by Henry *et al*. (2002).

The differences among the cryoprotectants are

especially due to the permeability coefficient and the structural model of the cryogenic agent. The most important characteristics of a cryoprotectant include its colligative properties, water affinity, and the ability to form hydrogen bonds. These interactions with water decrease the intracellular cryoscopy point by increasing the amount of water that remains liquid at low temperatures and reducing the intracellular concentration of solutes and the damages caused by the solution effect (Dalimata and Graham, 1997; Holt, 2000b; Watson, 2000). In addition to a low molecular weight and a low cellular toxicity, the cryogenic agent must have a great number of functional groups that are capable of forming hydrogen bonds with water. Ethylene glycol has the ability to form four hydrogen bonds with water, while acetamide can form only three bonds. EG is more capable of forming hydrogen bonds with water, while AC has a lower molecular weight (59.07; Syres, 1989), indicating that these biochemical characteristics probably provide compensatory effects and contribute to balancing the cryoprotective effectiveness of both cryoagents used in this study.

Acetamide alone is toxic and has a poor cryoprotective effect on stallion semen according to Graham (2000) and Squires *et al*. (2004). However, the incorporation of methyl cellulose and trehalose to the freezing extender resulted in enhanced post-thaw viability, as shown in this study. The property of the methyl cellulose and the capacity of the trehalose to interact with the lipids and proteins in the membrane (Holt, 1997, 2000a), which is important for membrane stabilization (Beattie *et al*., 1997; Ishida *et al*., 1997), may increase the ability of equine sperm to withstand damage after cryopreservation using acetamide.

Utilization of a freezing curve with or without prior cooling had no effect on post-thaw sperm motility or on the structural and functional integrity of the sperm membranes when analyzed independently of the other factors studied (cryoprotectants, thawing temperature and the interaction among cryoprotectants and freezing curves) ($P > 0.05$). Several researchers (Cochran *et al*., 1984; Ecot *et al*., 2000; Hernández *et al*., 2000; Crockett *et al*., 2001; Lagares *et al*., 2001; Papa *et al*., 2001; Bueno *et al*., 2002; Terraciano *et al*., 2008) have also studied the effects of freezing curves on post-thaw sperm viability. The freezing curves classified as slow, moderate and fast have not caused a different frequency of damage to the sperm during the cryopreservation of equine semen.

Cooling the semen before fast freezing is important to minimize the detrimental effects of cryopreservation on the sperm membranes (Papa *et al*., 2001; Alvarenga *et al*., 2005). The results of the present experiment have not demonstrated that the rapid curve was more damaging to the membranes of the sperm than the freezing curve with prior cooling. However, there was a tendency for the cryopreserved sperm exposed to a cooling curve before freezing to have higher percentages of structural and functional sperm membrane integrity after thawing.

The choice of the ideal freezing curve depends on the composition of the extender to be used for cryopreservation (Bedford *et al*., 1995). It is believed that, depending on the composition of sugars, buffers, egg yolk and/or milk and cryogenic agent in the extender, there should be a trend toward improving post-thaw sperm quality using certain freezing rates. In this study, it was found that the semen frozen in LAC+5% AC showed greater structural membrane integrity when the gametes were slow cooled prior to fast freezing (curve > -60°C/min), which allowed a longer interaction between the sperm and the extender. However, sperm frozen in LAC 3.5% EG showed similar post-thaw sperm motilities and membrane integrities when subjected to fast freezing either with or without prior cooling.

The best cryoprotection effect, which was obtained when the semen was diluted in LAC+5% AC and frozen with prior cooling, is probably attributable to the fact that acetamide is a molecule pertaining to the chemical function of amides (Syres, 1989). This cryogenic agent is less capable of penetrating the sperm membrane than other agents, such as DMSO, ethylene glycol and propylene glycol (Amann and Pickett, 1987) as it requires more time to cross the sperm membranes and act on the sperm's structures. On the other hand, the LAC 3.5% EG extender tended to preserve the motility and the functional and structural integrity of the sperm membrane better when the semen was subjected to freezing without previous slow cooling. This finding is probably due to the fact that ethylene glycol is a highly hydrophilic molecule in which the proportion of carbon atoms (C) and hydroxyl (OH) molecules is 1:1, and the low proportion of C:OH allows a high hydrophilicity and greater effectiveness in cryoprotection (Storey *et al*., 1998). In addition, the molecular weight of ethylene glycol (62.07) is inferior to those of glycerol (92.10), propylene glycol (76.10) and DMSO (78.13), thereby enabling higher permeability compared to the other cryogenic agents pertaining to the chemical function of alcohol (Gordon, 1996).

The ideal heating rate for thawing equine semen is influenced by the type of package (aluminium tubes, macrotubes, pellets and straws) and the time the sperm remained exposed to the extender during the cryopreservation process (Pickett and Amann, 1993). In this experiment, the effects of the package type and their interaction with the thawing temperatures were not studied; also, no interaction was observed between the extenders and the heating temperatures. The thawing temperature in a double boiler at 75°C for 7 s followed by immersion in a water bath at 37°C for 5 s has influenced the results of sperm motility and integrity, as assessed by fluorescence dyes ($P < 0.05$); the results were higher than when thawing occurred at 37°C, regardless of the cryoprotectant added to the extender

and the cooling protocol used. No effect of the thawing temperatures on the functional integrity of the sperm membranes was observed.

Heating using a fast curve is necessary to achieve better sperm survival results after thawing. High temperatures prevent the occurrence of crystallization in which the microscopic crystals of ice formed during freezing form larger crystals during slow thawing, thereby causing physical damage to the cells (Holt, 2000a). The thawing temperature of 37ºC for semen can expose the sperm membranes to temperature changes that lead to the reorganization of lipids or the movement of proteins. These changes are more severe than the changes that occur when a thawing temperature of 70ºC is used (Peña and Linde-Forsberg, 2000).

Using equine semen, Jasko (1994) demonstrated that thawing at 75ºC was better than thawing at lower temperatures. The danger of the elevated boiler temperature implies serious injuries to the spermatozoa if the 7 s time limit for immersion stipulated in the protocol is exceeded. In this study, cell death occurred when the samples were carelessly left for more than 8 s in a boiler at 75ºC.

Independently of the extender used, we observed great variability among the stallions in the response to sperm freezing. This high variability between individuals has been reported in other studies with stallions (Vidament *et al*., 1997; Henry *et al*, 2002; Alvarenga *et al*., 2005) and also in other species (Martinez-Pastor *et al*., 2005; Dorado *et al*., 2007; Fraser and Strzezek, 2007; Andrabi, 2009; Lopes *et al*., 2009).

Artificial insemination with frozen semen is the most efficient method for *in vivo* analysis to infer the fertile potential of sperm. However, the analysis requires a large number of animals to be evaluated and also depends on a range of factors that extend well beyond post-thaw sperm quality. Rousset *et al*. (1987) have described that at least 6 yr are needed to obtain a conclusive result for the evaluation of the fertility potential of frozen equine semen. The fertility tests should also be performed *in vivo* with the largest possible number of females.

The fertility of equine semen frozen using EG as a cryogenic agent has been tested by Neves Neto *et al*. (1995). The per-cycle overall pregnancy rate of 23.3% obtained in the present experiment using LAC+5% AC was considered unsatisfactory but opens a new avenue of research in the search for improvements to create alternative extenders for freezing equine semen. This rate was slightly lower than the rates reported by authors in previous studies (Palmer and Magistrini, 1992; Barbacini *et al*., 1999; Alvarenga *et al*., 2001; Juliani *et al*., 2002; Squires *et al*., 2002; Vidament *et al*., 2002; Moffet *et al*., 2003). Despite the low pregnancy rate, this rate was comparable to the per-cycle fertility rates reported in the literature for frozen equine semen, which ranged from 10 to 35% based on rectal palpation 50 days after ovulation (Cochran *et al*., 1983); 8 to 61% (Amann and Pickett, 1987) and 32 to 73% (Loomis, 2001) using glycerol as a cryogenic agent. When comparing fertility data, we must take into consideration variables such as freezing methods, frequency of insemination, sperm concentration, insemination dose, time of insemination with respect to the ovulation period of the mare, type of cryogenic agent used for freezing and other factors. All of these factors hinder our ability to compare the conception rates obtained in different studies with frozen equine semen.

In conclusion, this study showed that both EG or AC with methyl cellulose and trehalose similarly protected stallion sperm from cryodamage, that slow cooling to 5ºC before freezing is required when using LAC + 5% AC, and that thawing at 75ºC was better than at 37ºC. Also, it was evident that equine sperm frozen in an egg yolk-EDTA–glucose extender with acetamide, methyl cellulose and trehalose used as a replacement for glycerol are capable of achieving fertilization in mares.

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