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COMPUTERIZED ANALYSIS OF SPERM MOTION: EFFECTS OF GLYCEROL CONCENTRATION ON THE CRYOPRESERVATION OF EQUINE SPERMATOZOA

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SUMMARY

An experiment was conducted to examine the effects of glycerol added to a glucose-sucrose-dried skim milk freezing extender (B4) at 0, 1, 2, or 4% for cryopreservation of equine spermatozoa. Two ejaculates from each of three stallions were used in the study. Following cryopreservation and storage in liquid nitrogen for at least 30 days, the semen was thawed and subsequently diluted to approximately 33 x 10^6 sperm/ml in the freezing extender minus glycerol and egg yolk at 30°C. It was then incubated for 1 h at room temperature prior to evaluation. Each semen sample was evaluated for the percentage of motile sperm (M, %), the percentage progressively motile sperm (PM, %), velocity (V, μ/sec), progressive velocity (PV, μ/sec), linearity (L, %) and lateral head displacement (LHD, μ) using computer-aided sperm analysis (CASA). The data were statistically analyzed and the optimal glycerol concentration was determined for each parameter. For three of the parameters (M, PM and LHD), the 2% level of glycerol was selected by the statistical analysis as optimal ($P < .05$), while for the parameters (V and PV), the 1% level of glycerol was selected as optimal ($P < .05$). Lastly, linearity was highest in sperm frozen without glycerol and decreased ($P < .05$) with glycerol addition. Therefore, based on the results of the motion characteristics examined in the present study, we suggest that a 2% level of glycerol appears to provide maximum cryoprotection with a minimum potential for detrimental effects in stallion semen frozen as described herein.

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INTRODUCTION

Since Smith and Polge[1] first reported that glycerol could provide considerable protection to stallion spermatozoa during freezing and thawing, glycerol has repeatedly been demonstrated to be the most suitable cryoprotective agent for freezing stallion semen.[2] However, because of evidence that the presence of glycerol in extenders can markedly lower the fertility of spermatozoa of the stallion,[3] boar,[4] and ram,[5] studies to determine its lowest effective concentration should be examined in formulating any cryoprotective diluent.[6]

Traditional methods for sperm motion assessment have been limited to subjective visual observations via light microscopy which are fraught with high variability. The availability of computer-aided sperm analysis (CASA) to analyze sperm motion has provided an objective means to obtain accurate quantitative information on sperm motion variables. In the present study we have used CASA to evaluate the effects of glycerol concentration on sperm motion characteristics following cryopreservation of equine spermatozoa.

MATERIALS AND METHODS

Extender Preparation. The freezing extender (B4) consisted of glucose[a] 0.9 g; sucrose[a] 7 g; nonfat dry milk[b] 2.4 g; and 100 mg Ticarcillin[c] diluted to 100 ml in sterile water. The centrifugation extender contained 3 g glucose, 5 g sucrose, 1.5 g bovine serum albumin,[a] and 100 mg Ticarcillin diluted to 100 ml in sterile water. A clarified egg yolk solution containing 50% yolk was prepared by centrifugation of 25 ml yolk with 25 ml of centrifugation extender at 10,000 x g for 15 min at room temperature. The supernatant including lipid on the surface was pipetted to another tube and mixed well. The concentrated egg yolk solution was added to the B4 extender so that the extended semen contained a final concentration of 4% yolk. Glycerol[a] was added so that the extended semen contained a final concentration of 0, 1, 2, or 4 %. The osmotic pressure and pH for the B4 and centrifugation extenders were 360 and 350 mOsm/kg and 6.7 and 6.8, respectively.

Sperm Preparation. Extragonadal sperm reserves of

[a]Sigma Chem. Co., St. Louis, MO.
[b]Sanalac, Beatrice Foods Fullerton, CA.
[c]Ticar, Beecham Laboratories, Bristol, TN.

Table 1. Mean ± SE values for motion characteristics of cryopreserved stallion spermatozoa in B4 extender containing various concentrations of glycerol.

Glycerol	M, %	PM, %	V, μ/sec	PV, μ/sec	L, %	LHD, μ
0%	15.3±1.0	12.3±0.8	61.2±1.7	54.7±1.5	86.5±0.7*	4.4±0.1
1%	33.9±1.1	25.2±0.8	71.8±1.2*	61.7±1.1*	82.2±0.7	5.4±1.0
2%	42.5±1.4*	31.4±1.0*	76.2±1.1	64.9±1.0	81.6±0.5	5.8±0.1*
4%	44.6±1.5	32.8±1.0	75.1±1.2	63.7±1.2	80.6±0.5	5.8±0.1

*Selected as optimal glycerol concentration by the statistical analysis which selects the highest level associated with a significant (P<.05) increase in the characteristic studied.

the stallions were stabilized by daily semen collections for 3 days before the onset of the study. Two ejaculates from 3 stallions were collected by artificial vagina with a disposable liner with an in-line filter. Ejaculates were extended 2:1 (v/v) with centrifugation extender for centrifugation at 350 g for 5 min in 50 ml disposable centrifugation tubes (Falcon, catalog #2098)[d] followed by aspiration and disposal of the supernatant (note: because this centrifugation procedure usually results in a loose pellet, only the top 85 to 90% of the supernatant is removed leaving 5 to 7.5 ml containing the pellet). After centrifugation, the sperm were diluted to a final concentrations of 150 x 10^6/ml in each of the freezing extenders.

Freezing and Thawing Procedures. Sperm were frozen in a nitrogen vapor freezing unit[e] in 5 x 280-mm plastic straws[f] in static nitrogen vapor 1 cm above liquid nitrogen for 15 min. They were then plunged into liquid nitrogen for at least 10 min prior to transfer to a nitrogen storage tank. The straws were thawed for 52 sec in a tall cylindrical container (300 mm x 120 mm) filled with water at 52°C.

Evaluation of Sperm. Sperm motility, progressive motility, velocity, progressive velocity, linearity and lateral head displacement were evaluated using a Hamilton-Thorn Motility Analyze®;[g] equipped with negative phase contrast illumination. After thawing, samples were diluted 1:4 to a concentration of approximately 30 x 10^6 sperm/ml in B4 extender minus egg yolk and glycerol at 30°C and incubated for 1 h at room temperature prior to evaluation. Before evaluation, all samples were well mixed and a 5-μl drop of sperm suspension was placed on a pre-warmed counting chamber[h] and covered with a gridless coverslip. Following a 1-min equilibration period, three fields per drop were examined and 3 drops were examined from each sample. Settings for the HTM 2030 were as follows: frames acquired, 20; acquisition rate, 30 frames/sec; specimen dilution, none; layer thickness, 10 um; temperature 37°C; critical path velocity, 70; critical linear index, 70; slow cells motile, no; slow velocity gate, 20; minimal contrast, 9; minimal size, 7; low size gate, 0.5; high size gate 1.8; low intensity gate, 0.5; high intensity gate, 1.8; default pixel count, 10; and default intensity, 140.

Statistical Analysis. The data were analyzed using the SAS GLM procedure to conduct a mixed model ANOVA. Stallion, glycerol concentration, and stallion by glycerol concentration were tested using TEST statements with appropriate error terms. The optimal concentration was determined using orthogonal contrasts to compare each concentration of glycerol to all higher concentrations. The statistical analysis selected the highest level associated with a significant increase as the optimal concentration for each of the motion characteristics studied.

RESULTS

The motion analysis data for all ejaculates studied are presented in Table 1. For three of the parameters (M, PM and LHD), the 2% level of glycerol was selected as optimal, while for the parameters V and PV, the 1% level of glycerol was selected as optimal. Lastly, linearity was highest in sperm frozen without glycerol and decreased (P< .05) with glycerol addition.

DISCUSSION

Previous studies in our laboratory using a similar cryopreservation protocol that used a 3.5% level of glycerol reported significant 25% to 35% losses in sperm motility and velocity after cryopreservation and thawing.[7-9] Several observations from the present study suggest that a lower concentration of glycerol may be beneficial. First of

[d]Becton Dickinson Labware, Lincoln Park, NJ.
[e]Brazos Technologies, College Station, TX.
[f]Mini Tube, Madison, WI.
[g]HTM 2030, Hamilton-Thorn Research, Beverly, MA.
[h]Sefi-Medical Instruments Ltd., Haifa, Israel.

all, based on motility and progressive motility, glycerol levels greater than 2% offered no statistical improvement in these commonly studied endpoints. Also, lateral head displacement, which corresponds to the mean width of the head oscillation as the sperms swims, was not improved after glycerol levels reached 2%. Statistical examination of the data for velocity and progressive velocity indicated that swimming speeds were not improved after glycerol levels reached 1% and linearity, a measure of straightness, was highest in sperm frozen without glycerol and significantly decreased with glycerol addition. Therefore, glycerol levels greater than 2% offered no statistical improvement in cryoprotection for any of the motion parameters studied. The observation that addition of stallion sperm to a freezing medium containing only 2.5% glycerol caused significant reductions in mean track speed, LHD, percent normal spermatozoa and increased the proportions of spermatozoa with wrinkled acrosomal membranes, but did not significantly change motility prior to cryopreservation,[10] supports the idea of reducing glycerol concentrations in extenders for freezing stallion semen. However, such observations are based only on motion characteristics or sperm morphology and need to be confirmed in fertility trials.

In humans, several studies attempting to relate CASA measurements to fertility in cryopreserved ejaculates[11-13] demonstrated that velocity was statistically associated with fertility, while the more commonly used measurements of motility and progressive motility were found not to be reliable predictors of fertility. Such data suggest that motility measurements may have greater significance applied to them than appropriate in relation to the potential fertility of cryopreserved semen. Furthermore, velocity of cryopreserved sperm was the only motion characteristic correlated with a sperm population with functional mitochondria and intact plasma membranes identified by flow cytometry.[14] This suggests that membrane integrity is required for sperm to maintain adequate swimming velocity or that membrane integrity and mitochondrial function decrease at a rate similar to that of sperm velocity. Because cryopreservation has been consistently associated with decreased sperm velocity in humans,[15,16] cats,[17] and stallions,[7-10] methodologies for developing optimal procedures for freezing semen should strive to reduce or prevent such losses in velocity.

Recently, Padilla and Foote[18] demonstrated that supplementation of Kenney's extender with a high-potassium Tyrodes medium was very effective in preventing the decrease in velocity associated with slow-cooling centrifuged stallion semen. This high-potassium Tyrodes supplement should be examined for its effectiveness as a supplement in cryopreservation extenders like B4 used in the present study or a modified Kenney's extender which has been used for cryopreservation of stallion semen.[9] Our finding that velocity and progressive velocity were not statistically improved by glycerol levels higher than 1% indicates such studies should include groups with 1 and 2% levels of glycerol.

Glycerol is an essential cryoprotectant in all conventional extenders used for freezing stallion semen in concentrations between 2.5 and 6%.[2] Its concentration was usually determined using post-thaw motility, often in the presence of the glycerol. However, removal of human sperm[19,20] and boar sperm[21,22] from glycerol appears to result in osmotic damage which may, in turn, reduce the sperm's fertile life span. This may explain the observations of Abdelhakeam, Graham and Vazquez,[5] who demonstrated that incubation of ram semen for six hours at 5° C with and without 3% glycerol prior to insemination resulted in 41% and 83% lambing rates, respectively. Similar results were obtained in stallions where incubation of sperm cooled to 5°C over two hours in a cream-gel extender with and without 7% glycerol prior to insemination resulted in first cycle pregnancy rates of 5.6% and 38.9% and 3 cycle pregnancy rates of 44.4% and 83.3%.[3] Such decreases in fertility are similar to those associated with cryopreservation and point to the need for additional studies to optimize glycerol levels used in current cryopreservation protocols. It should be noted that the sperm cells in the present study were exposed to the osmotic stress of rapid glycerol removal by dilution (1:4) in B4 extender without egg yolk and glycerol.

Recent studies using cryomicroscopy have produced a number of unexpected and novel findings which suggest that sperm plasma membrane integrity is retained throughout the cooling and freezing process, even in the absence of cryoprotectants. These studies suggested that membrane damage was not manifested immediately after thawing, but occurred during post-thaw rewarming within specific temperature ranges between 2°C and 30°C, and may involve changes in membrane lipid organization or protein conformation.[23,24] Such observations suggest that it is the restoration of spermatozoa to isotonic equilibrium and physiological temperatures that is responsible for much of the membrane damage associated with cryopreservation. Therefore, research into better thawing and post-thawing semen handling procedures as well as extender development with

a focus on membrane stabilizing compounds[10,23-26] and reduction or elimination of toxic penetrating cryoprotectants like glycerol[27] are likely to improve the fertility of cryopreserved semen. In fact, this approach has recently resulted in a 52% lambing rate after cervical insemination of ram semen frozen in the absence of glycerol[5]. In addition, these same workers were only able to obtain lambing rates of 33% when semen was frozen in extender containing 3% glycerol and slow removal of the glycerol by dialysis after thawing increased the lambing rate to 48%.[5]

CONCLUSIONS

Based on our results, we suggest that a 2% level of glycerol appears to provide maximum cryoprotection with a minimum potential for detrimental effects for stallion semen frozen in B4 extender under the conditions of this study. However, our conclusion is based only on motion characteristics so that fertility trials remain necessary as the ultimate test of improved cryopreservation.

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COMPUTERIZED ANALYSIS OF SPERM MOTION: EFFECTS OF EGG YOLK CONCENTRATION ON THE CRYOPRESERVATION OF EQUINE SPERMATOZOA

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Bogart and Mayer (1950) published the first detailed studies on stallion semen extension. In their studies they noted that stallion spermatozoa had little inherent resistance to adverse conditions such as changes in pH, osmotic pressure or temperature. Furthermore, they reported that egg yolk contained a "spermatozoan resistance factor" capable of protecting stallion sperm from many of the harmful conditions listed above. Masuda and Nishikawa (1972) reported removal of ions and other dialyzable materials from egg yolk produced a yolk of a superior quality and that the low molecular weight materials were detrimental on sperm motility. Moreover, Nishikawa noted that seminal plasma removal and egg yolk addition resulted in increased sperm viability after freezing (Nishikawa, 1972). The level of egg yolk most commonly used by Nishikawa (1972) ranged between 2 to 5% although as little as 0.5% could be used. Since these early studies egg yolk has been included in almost every freezing extender reported (Amann and Pickett,1978). However, the concentrations have varied widely, ranging between 2% and 20%. Because egg yolk is considered a major cryoprotectant (Pace and Gram, 1974) and can influence the amount of glycerol required in freezing extenders (Pace and Gram, 1974; Watson and Martin, 1975) studies to determine its optimal concentration are important in the development of any extender to be used for cryopreservation of sperm cells.

The availability of computer-aided sperm analysis (CASA) to analyze sperm motion has provided an objective means to obtain accurate quantitative information on sperm motion variables. In the present study we have used CASA to examine effects of differing the concentrations of egg yolk added to the modified Kenney's freezing extender (B4) previously used for cryopreservation of stallion (Burns and Fleming, 1988) and Grevy's zebra spermatozoa (Crump and Crump, 1994) containing 4 and 10% egg yolk, respectively.

This experiment examined the effects of egg yolk added to a freezing extender at: 0, 2.5, 5 or 10% for cryopreservation of equine spermatozoa. Extragonadal sperm reserves of the stallions were stabilized by three daily semen collections before the onset of the study. Two ejaculates from 3 stallions were collected by artificial vagina with a disposable liner with an in-line filter. Next ejaculates were extended 2 to 1 with centrifugation extender (CE) for centrifugation at 350 g for 5 minutes followed by aspiration and disposal of approximately 80 % of the supernatant. The (CE) contained 5 g sucrose (S 1888); 3 g glucose (G 7021) ; 1.5 g bovine serum albumin (A 6793) (Sigma , St. Louis, MO) and 100 mg Ticarcillin (Ticar, Beecham, Bristol, TN) diluted to 100 mls in sterile water. Sperm were frozen in 5 x 180 mm plastic straws (Mini Tube. Madison, WI) in static nitrogen vapor 1 cm above liquid nitrogen for 15 minutes using a nitrogen vapor freezing unit (Brazos Technologies, College Station, TX). The freezing extender (FE) used contained glucose 0.9 g; sucrose 7 g; nonfat dry milk 2.4 g (Sanalac. Beatrice Foods Fullerton CA); and 100 mg ticarcillin diluted to 100 mls in sterile water. The osmotic pressure and pH for FE and CE were 360 and 350 mOsm/kg and 6.7 and 6.8, respectively. Glycerol was added so that the extended semen contained a final concentration of 3.5 %. The four different concentrations of egg yolk were added as a clarified concentrated egg yolk solution so that the extended semen contained a final concentration of 0, 2.5, 5, or 10% yolk (prepared by centrifugation of 25 ml yolk with 25 ml centrifugation extender at 10,000 x G for 15 minutes). Following cryopreservation and storage in liquid nitrogen for at least 30 days, straws were thawed in a tall cylindrical container filled with hot water at 52° C for 52 seconds. Samples were diluted to approximately 33 x 10^6 sperm/ml in the freezing extender minus glycerol and egg yolk at 30°C and incubated for one hour at room temperature prior to evaluation. Before evaluation all samples were well mixed and a 5 ul drop of sperm suspension was placed on a pre-warmed Makler chamber (Sefi-Medical Instruments Ltd., Haifa Israel) with a coverslip.

Following a one minute equilibration period three fields were examined from the first drop. This procedure was repeated for 3 additional drops so a total of 12 fields per sample were examined. Sperm were evaluated for the percentage of motile sperm (M%), progressively motile (PM %), velocity in microns per second (V m/sec), progressive velocity (PV m/sec), linearity (L %) and lateral head displacement in microns (LHD) using a Hamilton-Thorn Motility Analyzer (HTM 2030, Hamilton-Thorn Research, Beverly MA) equipped with negative phase contrast illumination. Settings for the HTM 2030 were as follows: frames acquired, 20; acquisition rate, 30 frames/sec; layer thickness, 10 um; temperature 37°C; slow cells motile (< 20 m/ sec , no). Data were statistically analyzed using the SAS GLM procedure to conduct a mixed model ANOVA. Stallion, yolk concentration, and stallion by yolk concentration were tested using TEST statements with appropriate error terms. The optimal concentration was determined using orthogonal contrasts to compare each concentration of yolk to all higher concentrations and then select the highest level associated with a significant increase in the characteristics studied. Motion analysis data for all ejaculate studied are presented in Table 1. For the parameters (M, PM and LHD) the 2.5% level of yolk was selected by the statistical analysis as optimal while the remaining parameters (V, PV and L) were not statistically improved by the addition of yolk.

Table 1. Mean±SE values for motion characteristics of cryopreserved stallion spermatozoa in B4 extender containing various concentrations of egg yolk.

Glycerol	M %	PM %	V m/sec	PV m/sec	L %	LHD m
0%	24.8±1.5	19 1±1.2	81.1±1.5*	69.0±1.7*	81 6±0.9*	5.5±0.1
2.5%	42.1±1.7*	31.2±1.2*	78.6±1.1	66.9±1.2	81.4±0.6	6.1±0.1*
5.0%	42.5±1.5	32.9±10	78.7±1.1	68.1±1.1	83.3±0.6	6.2±0.1
10.0%	43.5±1.7	33.7±1.2	76.8±1.5	66.7±1.4	83.1±0.6	5.9±0.1

* Selected as optimal egg yolk concentration by the statistical analysis which selects the highest level associated with a significant increase in the characteristic studied.

Therefore, based on the results of the present study we suggest that a 2.5 % level of yolk appears to provide maximum cryoprotection with a minimum of potential detrimental effects for stallion semen frozen as described above.

Key Words: stallion, semen, cryopreservation, egg yolk, motion characteristics

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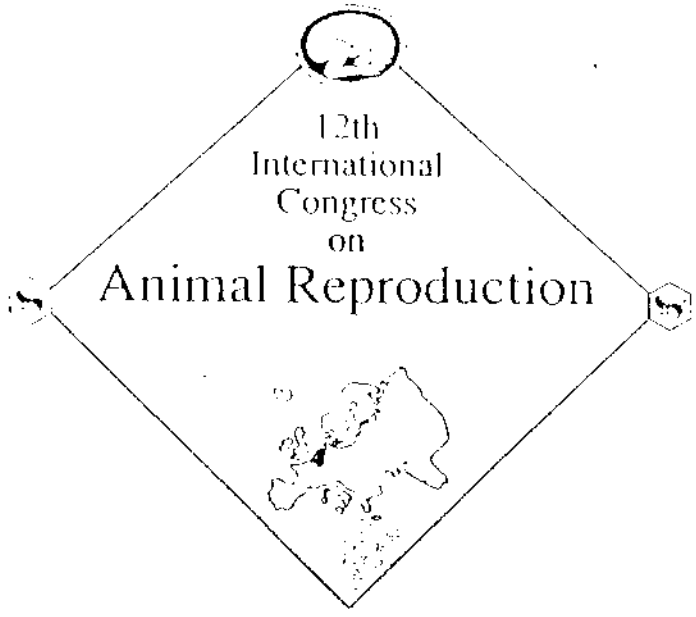

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MODIFICATION OF KENNEY'S EXTENDER FOR CRYOPRESERVATION OF EQUINE SPERMATOZOA

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SUMMARY

An experiment was conducted to examine the effects of using Kenney's extender containing glucose and nonfat dry milk or modifying it by substituting glucose with different cryoprotective sugars including sucrose, lactose raffinose trehalose or the polyol inositol plus the standard cryoprotectants glycerol (3.5%) and egg yolk (4%) for cryopreservation of equine spermatozoa. Following cryopreservation the percentage of motile sperm and velocity were evaluated objectively using a (HTM 2030) sperm motility analyzer. No significant differences were observed between glucose and the other sugars studied.

INTRODUCTION

Kenney et al. (1) found that equine sperm survived well in a powdered skim milk, glucose and gelatin extender and that it was superior to 5.6% glucose or 5.6% glucose and 5% egg yolk. This extender was slightly modified later when the gelatin portion of the formula was removed (2). More recently this extender has been shown to maintain excellent motility and fertilizing capacity of sperm cooled to 4°C for periods ranging from 12 to 72 hours (3-6). Based on these observations Burns and Fleming (3) modified this extender by replacing some of the glucose with the nonpenetrating cryoprotective sugar sucrose and adding glycerol (3.5%) and egg yolk (4%) for the successful cryopreservation of stallion semen. The present study was designed to examine if such sugars are of value compared to glucose and if so which ones may be best for cryopreservation of stallion semen.

MATERIALS AND METHODS

Extender Preparation. The six freezing extenders used contained nonfat dry milk 2.4 g (Sanalac, Beatrice Foods Fullerton CA); clarified egg yolk solution 8 ml (prepared by centrifugation of 25 ml yolk with 25 ml centrifugation extender at 10,000 x G for 15 minutes); 100 mg ticarcillin (Ticar, Beecham, Bristol, TN) and one of the following sugars glucose 4.9 g; sucrose 9.3; lactose 9.3 g; raffinose 16.2 g; trehalose 10.2 or inositol 4.9 g were diluted to 100 ml in sterile water. Glycerol was added in the extended semen at a final concentration of 3.5%. The centrifugation extender contained 5 g sucrose; 3 g glucose ; 1.5 g bovine serum albumin and 100 mg Ticarcillin diluted to 100 ml in sterile water. The osmotic pressure and pH for the freezing and

centrifugation extenders were 360 and 350 mOsm/kg and 6.7 and 6.8, respectively.

Sperm Preparation. Extragonadal sperm reserves of the stallions were stabilized by three daily semen collections before the onset of the study. Two ejaculates from 3 stallions used were collected by artificial vagina with a disposable liner with an in-line filter. Next ejaculates were extended 2 to 1 with centrifugation extender for centrifugation at 350 g for 5 minutes followed by aspiration and disposal of the supernatant. After centrifugation the sperm were diluted to a final concentration of 100 x 10^6/ml in each of the freezing extenders and sperm motility and velocity determinations were made as described below.

Freezing and Thawing Procedures. Sperm were frozen in 5 x 180 mm plastic straws (5 ml) in static nitrogen vapor 1 cm above liquid nitrogen for 15 minutes. The straws were thawed in a tall cylindrical container filled with hot water at 52°C for 52 seconds.

Evaluation of Sperm. Sperm were evaluated for the percentage of motile sperm and path velocity using a Hamilton-Thorn Motility Analyzer (HTM 2030; Hamilton-Thorn Research, Beverly MA) equipped with negative phase contrast illumination. Samples were diluted to 25 x 10^6 sperm/ml in their corresponding extender at 30°C and incubated for one hour at room temperature prior to evaluation. Before evaluation all samples were well mixed and a 5 ul drop of sperm suspension was placed on a pre warmed Makler chamber and covered with a gridless coverslip. Following a one minute equilibration period three fields per drop were examined and 3 drops were examined from each sample. Settings for the HTM 2030 were as follows: frames acquired, 20; acquisition rate, 30 frames/sec; specimen dilution, none; layer thickness, 10 um; temperature 37°C; critical path velocity, 70;critical linear index, 70; slow cells motile, no; slow velocity gate, 20; minimal contrast, 9; minimal size, 7; low size gate, 0.5; high size gate 1.8; low intensity gate, 0.5; high intensity gate, 1.8; default pixel count, 10; and default intensity, 140.

RESULTS AND DISCUSSION

Prior to cryopreservation sperm motility and velocity measurements did not differ ($p>0.05$) between extenders and averaged 85% and 90.3 micron per second, respectively. After cryopreservation and thawing significant decreases ($p<0.05$) in motility and velocity were observed within each treatment however, no differences ($p>0.05$) were detected among any of the extenders studied for either motility or velocity after a one hour post thaw incubation period. The 20 to 30 percent loss of motility and velocity as a result of cryopreservation is similar to earlier studies in our laboratory using a similar skim milk extender containing both sucrose and glucose (3).

The observation that the nonpenetrating cryoprotective sugars did not enhance cryosurvival of stallions differs from the findings of Nagase et al. (8) where addition of high molecular weight sugars enhanced cryosurvival of bull spermatozoa. It may be that because the stallions used in this study all had excellent seminal parameters and were known to be good freezers, the potential value of the cryoprotective-sugars may have been underestimated. However, until the existence of such an effect can be demonstrated the use of Kenney's skim milk and glucose extender which is easy to make, readily available and provides excellent cryoprotection when supplemented with 3.5% glycerol and 4% egg yolk deserves further attention as an equine freezing extender.

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Burns

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1989 JOINT ANNUAL MEETING
AMERICAN DAIRY SCIENCE ASSOCIATION AND AMERICAN SOCIETY OF ANIMAL SCIENCE

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Abstract and Abstract Heading (see instruction sheet for example)

Computerized analysis of sperm motion: Effects of storage at 4°C on slow-cooled and cryopreserved equine spermatozoa. P. J. Burns* and S. A. Fleming, Thornbrook Farms, P.O. Box 421 Bedford Hills, NY 10507.

In the present study the Hamilton-Thorn Motility Analyzer (HTM-2030) was used to investigate the effects of storage at 4°C following slow-cooling (SC) of fresh and frozen-thawed (FT) equine spermatozoa from 4 stallions. Semen was extended 5 to 1 in Kenney's extender (K) and incubated for 1 hour at room temperature (1 HR RT) or packed in an equitainer (Hamilton-Thorn) for SC. The remainder of the ejaculate was frozen as previously described (J. Anim. Sci. 66:391 Suppl. 1, abstr. 413, 1988). Non-frozen semen samples were analyzed after the following periods of storage (1 HR RT); 24, 48, and 72 HRS at 4°C following SC (24 HR SC); (48 HR SC); (72 HR SC). Frozen samples were thawed and extended 5 to 1 in K and analyzed after 1 HR at RT (1 HR FT) or 24 HRS at 4°C following SC (24 HR FT-SC). All samples were incubated 1 HR at RT before evaluation. Parameters studied were: Motility, % (M); Progressive Motility, % (Mp); Velocity, microns/second (V); Progressive Velocity, microns/second (Vp); Linear Index, % (LI).

Sperm Motion Parameters (mean ± SE)

Storage Period	M	Mp	V	Vp	LI
1 HR RT	88.5±1.1	61.9±1.6	89.1±1.6	71.8±1.7	77.2±1.3
24 HR SC	84.8±1.3	58.9±1.3	83.2±2.5	65.7±2.0	77.0±1.0
48 HR SC	79.1±2.6	55.0±2.2	68.6±2.5	54.1±2.3	76.0±0.8
72 HR SC	72.8±2.5	47.5±2.1	63.4±2.0	47.5±1.9	72.7±1.0
1 HR FT	59.3±2.5	43.9±1.7	65.2±1.4	56.6±1.3	80.9±0.7
24 HR FT-SC	53.9±2.2	39.8±1.9	57.0±1.1	46.6±1.2	79.0±0.8

Our results indicate that SC semen was stored for 24 HRS with no reduction in M, Mp, V, Vp & LI while FT semen had significantly lower M, Mp, V & Vp. Lastly, 72 HR SC and 1 HR FT values for Mp, V & Vp were not different. (Key Words: Stallion, Semen, Cryopreservation)

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ABSTRACT AND ABSTRACT HEADING (see instruction sheet for example)

Computerized analysis of sperm motion: Effects of caffeine and theophylline on cryopreserved equine spermatozoa. P. J. Burns and S. A. Fleming, Thornbrook Farms, P. O. Box 421, Bedford Hills, NY 10507.

In the present study the Hamilton-Thorn Motility analyzer (HT-M2000) was used to investigate the effects of caffeine (C) and theophylline (T) on cryopreserved spermatozoa from 2 ejaculates from each of 4 stallions. Ejaculates were extended 1:1 in a extender containing sterile H_2O, 100 ml; glucose, 6 g; BSA, 1.5 g; (ph 6.7) for centrifugation at 400 g for 3 minutes. Following aspiration of the supernatant the freezing extender containing sterile H_2O, 100 ml; sucrose, 7 g; glucose, 0.9 g; sanalac 2.4 g; egg yolk, 4 ml; glycerol, 35 ml was added slowly to give a concentration of 200 x 10^6 sperm/ml. Sperm were frozen in 5 x 280 mm plastic straws (5 ml) in static N_2 Vapor for 15 min. 1 cm above the LN_2. After storage in LN_2 for at least 60 days the straws were thawed and diluted 10 fold in a skim milk glucose extender (K) containing (C) 10 uM; (T) 10 uM; or extender only (K) and incubated 1 hour before evaluation. Paremters studied were: Motility (M), Progressive Motility (Mp), Velocity (V), Progressive Velocity (Vp), and Linearity (L).

Percentage change* of semen parameters after cryopreservation

Treatment	M	Mp	V	Vp	L
K	-21.4±7.6	-0.5±6.5	-28.2±4.9	-10.2±10	+28.1±6.5
C	-3.3±7.0	+18.9±6.2	-20.0±6.7	-3.8±10.1	+24.9±5.0
T	-9.9±9.5	+13.8±8.7	-23.8±4.9	-1.1±8.8	+31.5±7.2

*(Prefreeze value - post-thaw value)/prefreeze value x 100

Our results indicate cryopreservation significantly reduced (M), (V), (Vp) and significantly increased (L). Also, (C) significantly increased (M) and (Mp) in frozen-thawed stallion semen.

KEY WORDS: Stallion Cryopreservation Semen Caffeine

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COMPUTERIZED ANALYSIS OF SPERM MOTION: EFFECTS OF RELAXIN ON CRYOPRESERVED EQUINE SPERMATOZOA. P. J. Burns and S. A. Fleming*, Thornbrook Farms, P. O. Box 421, Bedford Hills, NY 10507.

In the present study the Hamilton-Thorn Motility Analyzer (HT-M2030) was used to investigate the effects of relaxin on cryopreserved spermatozoa from 6 stallions. Ejaculates were extended 2 to 1 in an extender containing sterile H_2O, 100 ml; glucose, 3.0 g; sucrose, 5.0 g; BSA, 1.5 g; (ph 6.7) for centrifugation at 200 to 400 g for 4 to 7 minutes, depending on individual seminal characteristics. Following aspiration of the supernatent a freezing extender containing sterile H_2O, 100 ml; sucrose, 7 g; glucose, 0.9 g; sanalac, 2.4 g; egg yolk, 4 ml; and glycerol, 3.5 ml was added slowly to give a concentration of 125 x 10^6 sperm/ml. Sperm were frozen in 5 x 230 mm plastic straws (5 ml) in static N_2 vapor for 15 min. 1 cm above the LN_2. After storage in LN_2 for at least 60 days, straws were thawed and diluted 5 fold in a skim milk, glucose extender containing 400 ng. purified porcine relaxin (RXN-P1 NIDDK & NHPP) or extender only and incubated 1 hr. at room temp. before evaluation. Parameters studied were: Motility, % (M); Velocity, microns/second (V); Progressive Velocity, microns/second (Vp); Linear Index, % (LI).

	Sperm Motion Parameters (mean ± SE)			
Treatment	M	V	Vp	LI
Control	53.4±1.6	62.5±1.1	53.5±0.7	82.4±0.6
Relaxin	60.5±1.8	72.7±1.1	61.2±0.9	81.4±0.6

Our results indicate that Relaxin significantly increased M, V, and Vp in frozen thawed stallion semen.

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