Comparative Study of Some Permeating Cryoprotectants for Cryopreserving Bull Spermatozoa

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Abstract: Computer-assisted sperm analyzers (CASA) have become the standard tool for evaluating sperm motility because they provide objective results for thousands of mammalian spermatozoa. Mammalian spermatozoa experience osmotic stress when the glycerol is added to the cells prior to freezing and removal from the cells after thawing. In order to minimize osmotic damage, cryoprotectants having lower molecular weights and greater membrane permeability than glycerol, were evaluated to determine their effectiveness for cryopreserving bull spermatozoa. The aim of this study was to compare the cryopreservation effects of low molecular weight cryoprotectants (Ethylene glycol and Methanol) to glycerol, on post-thaw CASA sperm parameters. Bull semen was diluted with tris-egg yolk extender containing 3% glycerol, 3, 2 & 1% ethylene glycol or 3, 2 & 1% methanol. Bull semen was frozen in 0.25 pellets using the cold surface of cattle fat. Bull spermatozoa exhibited higher percentages ($P < 0.01$) for total (Mot, 72.4%) and progressively (Prog, 29.5%) motilities when frozen in extender containing 3% glycerol compared to 3, 2 & 1% ethylene glycol or 3, 2 & 1 % methanol. In conclusion, no advantages were found in using ethylene glycol or methanol to replace glycerol in bull semen freezing. Glycerol provided the best sperm characteristics for bull spermatozoa after freezing and thawing. The possibility of using ethylene glycol or methanol as permeating cryoprotectants for bull semen deserves further investigation, and these cryoprotectants should also be evaluated in extenders that do contain disaccharides or cholesterol.

Keywords: Cryopreservation, pellets, spermatozoa, bull, cryoprotectant

INTRODUCTION

Conventional methods for semen analysis, including semen motility evaluation, are subjective. Variations of 30–60% have been reported in subjective microscopic evaluations of human and animal semen in the same ejaculates (Amann, 1989; Budworth *et al.,* 1988; Auger *et al.,* 1993; Coetzee *et al.,* 1999). The availability of data recorded by CASA facilitates the comparison of results and makes it possible to find subtle differences between treatments (Verstegen *et al.,* 2002) Also, CASA systems appear to have high accuracy and repeatability (Davis *et al.,* 1992; Farrell *et al.,* 1995).

Most semen cryopreservation protocols still favor glycerol in the cryoprotective media, following the example set by Polge *et al.* (1949). Glycerol, together with methanol, and ethylene glycol belong to a group which permeate into the sperm cell. Glycerol is the most commonly used cryoprotectant for farm animal spermatozoa. However, glycerol when used in concentrations has some toxicity and also may be the cryoprotectant that causes the greatest osmotic damage to spermatozoa because its permeability across the sperm plasma membrane is much lower than that of water and lower than that of many other cryoprotectants (Guthrie *et al.,* 2002).

Low-molecular-weight cryoprotectants (Ethylene glycol and Methanol) may prove less damaging to spermatozoa, as they equilibrate across the plasma membrane more readily than glycerol and will therefore induce much smaller and potentially less damaging cell excursions than glycerol (Moore *et al.,* 2006). Squires *et al.* (2004) found that stallion spermatozoa cryopreserved using ethylene glycol had similar post-thaw spermatozoal attributes to spermatozoa cryopreserved with glycerol.

It was hypothesized that methanol could function as a cryoprotectant during freezing and thawing of equine embryos since it enters and leaves mammalian cells rapidly without notable changes in their volume (Pfaff, 1994) and protects embryos against deleterious effects of cryopreservation (Yamamoto et. al., 1982). Methanol was found to be effective cryoprotectants for sturgeon (Tsvetkova *et al.,* 1996; Glogowski *et al.,* 2002; Urbanyi *et al.,* 2003) and paddlefish (Mims *et al.,* 2000) sperm. Methanol has been used for the cryopreservation of cyprinid species such as zebrafish (Harvey *et al.,* 1982). Methanol is nontoxic when used as a cryoprotectant and was found to be superior to DMSO or glycerol in a number of cell types such as mammalian tissue-culture cells (Harvey *et al.,* 1982).

The aim of this study was to compare the cryopreservation effects of low molecular weight cryoprotectants (Ethylene glycol and Methanol) to glycerol, on post-thaw CASA sperm parameters.

MATERIALS AND METHODS

Animals:

Semen was collected from 9 bulls housed at Colorado State University, using an artificial vagina and the semen from each bull was separately processed for cryopreservation, immediately after collection.

Semen Collection, Processing, Cryopreservation:

All Chemicals used were reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO). Clarified egg yolk was also used to supplement bull semen extenders. Egg yolk particles can be misinterpreted as non-motile spermatozoa by CASA systems (Jasko *et al.,* 1992); therefore, after the addition of egg yolk to each of the extenders, the media were centrifuged at 10,000 x g for 25 minutes to remove the egg yolk particles. After centrifugation, the supernatant

was filtered through 3 filters of decreasing pore size: 5.0 µm, 3.0 µm and 0.8 µm, and 0.45 µm.

After collection, semen was assessed for the percentages of total and progressively motile spermatozoa and for sperm concentration. Visual motility estimates were made from a drop of semen diluted with Tris buffer on a pre-warmed slide. Each drop was evaluated for total and progressive motility at 400 x magnification using a phase contrast microscope. Samples containing greater than 70% total motile sperm were used in this study.

Each semen sample was diluted to 50 x 10^6 cells/ml of diluent A (Table 1) at 37°C and then divided into 7 aliquots. Each aliquot was cooled to 5°C over 2 hrs, at which time an equal volume of each treatment medium was added resulting in final concentration of glycerol is 3% (control), the final concentrations of ethylene glycol are 3%, 2% & 1% and the final concentrations of methanol are 3%, 2% & 1% (Table 1). The sperm were then equilibrated at 5°C over another 2 hours, then frozen in liquid nitrogen vapor (5 cm above the liquid nitrogen) using the cold surface of cattle fat (Awad and Graham, 2004) for 10 minutes prior to being plunged into liquid nitrogen for storage. Pellets were thawed in a water bath at 37°C for 1min using dry test tubes.

Semen Evaluation:

Samples were analyzed using a Hamilton Thorne Motility Analyzer (CASA; Hamilton-Thorn IVOS, Bedford, MA). CASA systems permit the evaluation of sperm motility in a relatively non-biased manner. These systems also permit the velocities of spermatozoa to be determined. The percentages of motile sperm in bull sperm samples were determined using a computer assisted sperm motion analysis system and a minimum of 200 spermatozoa per sample were evaluated. The settings of the CASA system included: 30 frames acquired at 60Hz; minimum contrast 20; minimum cell size 6; threshold straightness 60; medium average path velocity cutoff = 60m/s; low average path velocity cutoff = 25m/s; low straight line velocity cutoff = 10m/s; non-motile head size 17; non-motile head intensity 70. Data collected for these samples included the percentage of motile spermatozoa (Mot), the percentage of progressively motile spermatozoa (Prog), the average path velocity (VAP), the straight line velocity (VSL), the curvilinear velocity (VCL) and the linearity(LIN) of the motile cells.

Statistical Analysis:

Percentage data were transformed using arcsin. Differences in the percentages of total motile sperm (MOT), progressively motile sperm (PROG), the average path velocity (VAP), the straight line velocity (VSL), the curvilinear velocity (VCL) and the linearity (LIN) of the motile cells between the cryoprotective agents used, were determined by analysis of variance. Treatment differences were separated using Student–Newman–Keuls multiple range test (SAS, 1990).

RESULTS

Spermatozoa frozen in a medium which containing 3% glycerol exhibited the highest percentages ($p < 0.01$) of total (MOT: 72.38%), and progressive (PROG: 29.50%), motilities compared with ethylene glycol or methanol. Similarly, all different concentrations of ethylene glycol had higher values for MOT % and PROG % compared with methanol (Table 2 and Fig. 1& 2).

For the other parameters evaluated by CASA: VAP, VSL, VCL and Linearity (LIN) were affected by the type of cryoprotectant, Glycerol and ethylene glycol had higher values of VAP and VCL ($p<0.01$) than methanol. For VSL and LIN, only glycerol had higher values than ethylene glycol or methanol regardless the concentration of the cryoprotectant.

When bull spermatozoa were cryopreserved in medium containing 1, 2 or 3% ethylene glycol, no significance differences of CASA sperm parameters after freezing and thawing were found. Similarly, There were no significance differences of CASA sperm parameters after cryopreservation were found when bull semen cryopreserved in medium containing 1, 2 or 3% methanol.

Table (1): Composition of the media used for bull sperm cryopreservation.

	Media							
	Diluent A	***1***	***2***	***3***	***4***	***5***	***6***	7
Tris (mM)	250	250	250	250	250	250	250	250
Citric acid (mM)	88.5	88.5	88.5	88.5	88.5	88.5	88.5	88.5
Glucose (mM)	68.8	68.8	68.8	68.8	68.8	68.8	68.8	68.8
G % (v/v)	0	6	0	0	0	0	0	0
EG %(v/v)	0	0	6	4	2	0	0	0
M %(v/v)	0	0	0	0	0	6	4	2
EY % (v/v)	20	20	20	20	20	20	20	20

Table (2): The percentages of motile spermatozoa (Mot), progressively motile spermatozoa (Prog), average path velocity (VAP), average straight line velocity (VSL), curvilinear velocity (VCL) and linearity (LIN) when bull spermatozoa were frozen in 0.25 ml pellets using 7 different cryoprotective media (N = 8)

Media	MOT (%)	PROG (%)	VAP (um/s)	VSL (um/s)	VCL (um/s)	LIN
3% G	72.4[a]	29.5[a]	68.5[a]	59.6[a]	110.6[a]	55.5[a]
3% EG	56.9[b]	20.8[b]	68.2[a]	48.4[b]	112.4[a]	47.9[b]
2% EG	55.1[b]	21.9[b]	70.9[a]	47.4[b]	115.1[a]	47.1[b]
1% EG	44.5[c]	18.8[b]	67.5[a]	48.4[b]	114.7[a]	46.2[b]
3% M	22.6[d]	8.3[c]	59.4[b]	45.2[b]	105.7[b]	45.6[b]
2% M	15.9[d]	6.1[c]	57.4[b]	48.3[b]	92.4[b]	46.1[b]
1% M	14.5[d]	5.3[c]	55.9[b]	43.4[b]	98.0[b]	44.4[b]
SEM	3.1	1.4	1.4	1.4	2.5	1.1

[a,b] Means in the same column with different superscripts differ significantly at $p < 0.01$.

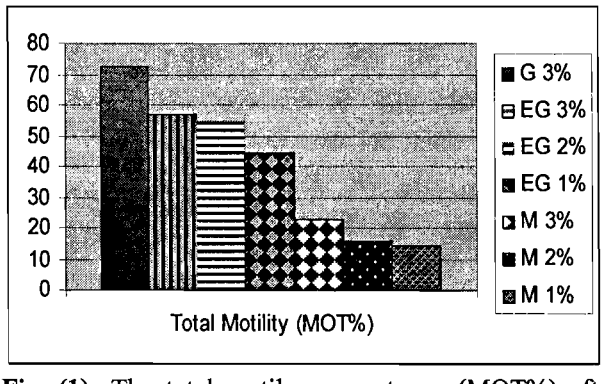

Fig. (1): The total motile spermatozoa (MOT%) after being cryopreserved in different media containing 3% glycerol (G), 3, 2 & 1% ethylene glycol (EG) and 3, 2 & 1% methanol (M).

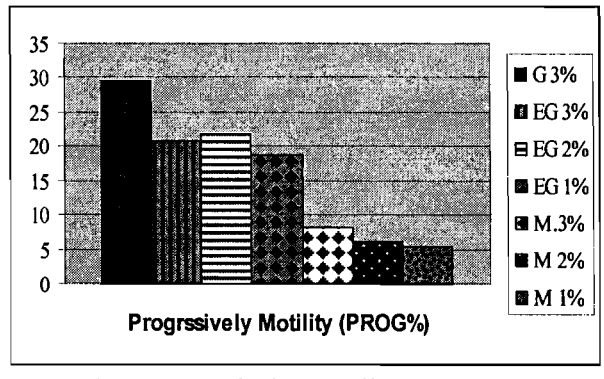

Fig. (2): Progressively motile bull spermatozoa (PROG%) after being cryopreserved in different media containing 3% glycerol (G), 3, 2 & 1% ethylene glycol (EG) and 3, 2 & 1% methanol (M).

DISCUSSION

Many factors affect sperm survival, e.g., the type of cryoprotective agents, and the concentration of cryoprotectants. Awad and Graham (2002) found that motility of bull spermatozoa was not negatively influenced by the reduction of glycerol concentration and no significant difference in the percentages of motile cells were found using a range of 2 to 6% glycerol. This study showed that no advantages were found in using ethylene glycol or methanol at any concentration to replace glycerol in bull semen freezing and glycerol provided the best CASA sperm parameters for bull spermatozoa after freezing and thawing. In contrast, the use of ethylene glycol for cryopreservation of stallion (Mantovani *et al.*, 2002; Henry *et al.*, 2002) or ram (Molinia *et al.*, 1994) semen can provide similar or better results than those obtained with glycerol. In this study, the extender with ethylene glycol, at any concentration, did not produce better results than the extender with glycerol, similarly to what was previously found by Cavalcanti *et al.* (2002) working with 4% and 7% ethylene glycol and 7% glycerol.

The effects of the ethylene glycol as a semen cryoprotectant may be species-specific (Ana Martins Bessa *et al.*, 2006). Some of the disadvantages of ethylene glycol may be due to deleterious effect on the motility of frozen spermatozoa (Alvarez and Storey, 1993). It has been also suggested that ethylene glycol could not reverse the sperm capacitation-like changes that occur in the freezing process (Rota *et al.*, 2006). Other differences contributing to the different effects of ethylene glycol versus glycerol may be the lower molecular weight of ethylene glycol, which allows it to penetrate and leave the cell faster than other cryoprotectants (Soares *et al.*, 2002). Similarly, the addition and removal of ethylene glycol seemed to minimize alterations of cellular volume of bovine spermatozoa, when compared with using glycerol (Gutherie *et al.*, 2002). However, permeability of the sperm cell membrane to either ethylene glycol or glycerol, and the consequent osmotic-induced alterations of cellular volume, may also be species-specific (Ana Martins Bessa *et al.*, 2006).

Post-thaw CASA sperm characteristics depended on the concentration of the cryoprotectant, lower concentration of methanol or ethylene glycol (1% or 2%) yielded lower post-thaw sperm characteristics which suggests that at lower concentrations it becomes not enough for sperm cryopreservation. Since cells are permeable to glycerol (Seidel, 1996) and methanol (Czlonkowska, *et al.*, 1991), cryoprotectants

immediately started to enter the embryo and continued to do so until equilibrium was reached. Methanol or ethylene glycol showed no advantage over glycerol in providing protection against the deleterious effects of cryopreservation and thawing of bull spermatozoa. Further studies are necessary to determine the efficacy of methanol or ethylene glycol at higher concentrations when used as a cryoprotectant for bull spermatozoa.

In conclusion, no advantages were found in using ethylene glycol or methanol to replace glycerol in bull semen freezing. Glycerol provided the best sperm characteristics for bull spermatozoa after freezing and thawing. The possibility of using ethylene glycol or methanol as permeating cryoprotectants for bull semen deserves further investigation, and these cryoprotectants should also be evaluated in extenders that do contain disaccharides or cholesterol.

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REFERENCES

Alvarez J. G. and B. T. Storey (1993). Evidence that membrane stress contributes more than lipid peroxidation to sub lethal cryodamage in cryopreserved human sperm: glycerol and other polyols as sole cryoprotectant. J Androl 14:199-209.

Amann R. P. (1989). Can the fertility potential of a seminal sample be predicted accurately? J Androl 10:89-98.

Martins-Bessa, A., A. Mayenco-Aguirre and A. Rocha (2006). Comparing ethylene glycol with glycerol for cryopreservation of canine semen in egg-yolk TRIS extenders. Theriogenology 66: 2047-2055.

Awad M. M., J. K. Graham (2002). Computerized motion analysis of bull spermatozoa cryopreserved in the presence of non-permeating and permeating cryoprotectants. Agric. Res. J. 1: 85-90.

Auger J, S. Leonce, P. Jouannet, X. Ronot (1993). Flow cytometric sorting of living, highly motile human spermatozoa based on evaluation of their mitochondrial activity. J Histochem Cytochem;41:1247-51.

Budworth P. R., R. P. Amann, P. L. Chapman (1988). Relationships between computerized measurements of motion of frozen thawed bull spermatozoa and fertility. J Androl 9:41-54.

Cavalcanti M. C. O., C. S. Moura, M. M. P. Guerra, S. V. Silva (2002). Acc¸ao crioprotectora do glicerol e etileno glicol no congelamento do semendecao (Cryoprotectoraction of the glycerol and ethylene glycol in the freezing of the dog semen).Rev Bras Reprod Anim 26 :174–6 [in Portuguese with English abstract].

Coetzee, K., T. F. Kruger, C. J. Lombard (1999). Repeatability and variance analysis on multiple computer-assisted (IVOS) sperm morphology readings. Andrologia 31:163-8.

Czlonkowska, M., K. Papis, A. Guszkiewicz, M. Kossakowski and U. Eysymont (1991). Freezing of sheep embryos in 30 M methanol. Cryoletters 12:11-16.

Davis R. O., S. A. Rothmann, J. W. Overstreet (1992). Accuracy and precision of computer-aided sperm analysis in multicenter studies. Fertil Steril 57:648 -53.

Duncan, D. B. (1955). Multiple Ranges and Multiple F-Test. Biometrics, 11:1-42.

Farrell P. B., V. Trouern-Trend, R. H. Foote and D. Douglas-Hamilton (1995). Repeatability of measurements on human, rabbit, and bull sperm by computer-assisted sperm analysis when comparing individual fields and means of 12 fields. Fertil Steril 64: 208-10.

Glogowski, R. Kolman, M. Szczepkowski, A. Horvath, B. Urbanyi, P. Sieczyn′ ski, A. Rzemieniecki, J. Domagała, W. Demianowicz, R. Kowalski, A. Ciereszko (2002). Fertilization rate of Siberian sturgeon (Acipenser baeri, Brandt) milt cryopreserved with methanol, Aquaculture 211 2002; 367-373.

Guthrie H. D., J. Liu, J. K. Critser (2002). Osmotic tolerance limits and effects of cryoprotectants on motility of bovine spermatozoa. Biol Reprod 67: 1811-1816.

Harvey, B., R. N. Kelley and M. J. Ashwood-Smith (1982). Cryopreservation of zebra fish spermatozoa using methanol. Can. J. Zool. 60:1867-1870.

Henry M, P. P. N. Snoeck, A. C. P. Cottorello (2002). Post-thaw spermatozoa plasma integrity and motility of stallion semen frozen with different cryoprotectants. Theriogenology 58 :245-8.

Jasko D. J., T. V. Little, D. H. Lein, R. H. Foote (1992). Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987- 1988). J. Am. Vet. Med. Assoc. 200, 979- 985.

Mantovani, R., A. Rota, M. E. Falomo, L. Bailoni, L. Vincenti (2002). Comparison between glycerol and ethylene glycol for the cryopreservation of equine spermatozoa: sperm quality assessment with standard analyses and with the hypoosmotic swelling test. Reprod Nutr Dev 42: 217-26.

Mims, S. D., L. I. Tsvetkova, G. G. Brown and B. I. Gomelsky (2000). Cryopreservation of sperm of sturgeon and paddlefish. In: Tiersch, T.R., Mazik, P.M. (Eds.), Cryopresertvation in Aquatic Species. World Aquaculture Society, Baton Rouge, USA, pp. 123-129.

Molinia F. C., G. Evans, W. M. C. Maxwell (1994). Incorporating of penetrating cryoprotectants in diluents for pellet-freezing ram spermatozoa. Theriogenology 42:849-858.

Moore A. I., E. L. Squires, J. E. Bruemmer and J. K. Graham (2006). Effect of cooling rate and cryoprotectant on the cryosurvival of equine

spermatozoa. Journal of Equine Veterinary Science May.

Pfaff, R. T. (1994). Effects of cryoprotectants on viability of equine embryos. MS Thesis, Colorado State University.

Polge, C., A. U. Smith and A. S. Parkes (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164: 666-676.

Rota A, C. Milani, G. Cabianca, M. Martini (2006). Comparison between glycerol and ethylene glycol for dog semen cryopreservation. Theriogenology 65:1848-1858.

SAS (1990). SAS User's Guide, Statistics. Statistical Analysis System Institute Inc., Cary, NC.

Seidel Jr, G. E. (1996). Cryopreservation of equine embryos. Vet Clin North Am: Equine Pract 12:85-99.

Soares M. P., C. A. R. Rossi, A. Mezzalira, M. Cecim (2002). Etileno glycol na criopreservac¸ao de semen canino (Ethylene glycol on canine semen cryopreservation). Ciencia Rural 32:649-55 [in Portuguese with English abstract].

Squires E. L., S. L. Keith, J. K. Graham (2004). Evaluation of alternative cryoprotectants for preserving stallion spermatozoa. Theriogenology 62:1056-1065.

Tsvetkova, L. I., J. Cosson, O. Linhart, R. Billard (1996). Motility and fertilizing capacity of fresh and frozen–thawed spermatozoa in sturgeons Acipenser baeri and A. ruthenus. J. Appl. Ichthyol. 12, 107-112.

Urbanyi, B., A. Horva´th, B. Kova´cs (2003). Successful hybridization of Acipenser species using cryopreserved sperm. Aquac. Int. 12, 47-56.

Verstegen, J., M. Iguer-Ouada, K. Onclin (2002). Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57:149 -79.

Yamamoto Y, N. Oguri, Y. Tsutsumi, Y. Hachinohe (1982). Experiments in the freezing and storage of equine embryos. J Reprod Fertil. 32 (Suppl):399-403.