11-2010

Use of Direct Thaw Insemination to Establish Pregnancies with Frozen–Thawed Semen from a Standard Jack

Rebecca J. Jepsen
United States Department of Agriculture

Lawrence E. Evans
Iowa State University

Curtis R. Youngs
Iowa State University, cryoungs@iastate.edu

Use of Direct Thaw Insemination to Establish Pregnancies with Frozen–Thawed Semen from a Standard Jack

Abstract
Pregnancy rates reported after artificial insemination with frozen–thawed jack spermatozoa have been relatively low compared with those attained in other species. Cholesterol is known to influence post-thaw fertility of both jack and stallion semen, and altering the amount of cholesterol in the freezing extender may help improve the fertility of frozen–thawed jack semen samples. In this study, we report clinical work that was performed using semen samples collected from a single jack. Samples were extended in EZ Mixin OF and then slowly cooled to 5°C. Extended semen samples were centrifuged at 400 × *g* for 10 minutes and the supernatant was discarded. Spermatozoa were resuspended in freezing medium to a final concentration of 400 × 106 cells/mL and were later frozen in liquid nitrogen vapor. Freezing extender treatments containing 2% ethylene glycol included the following: (1) 20% egg yolk (EY), (2) 5% EY, and (3) 20% EY + 60 mM hydroxypropyl-β-cyclodextrin (β-CD). For this study, a total of 28 mares aged 2 to 18 years was used over five breeding seasons (82 total cycles). Mares were administered human chorionic gonadotropin to induce ovulation when the dominant follicle was ≥35 mm in diameter. They were inseminated within 6 hours before ovulation and again within 6 hours after ovulation. Pregnancy rates obtained were as follows: (1) 6.25% (one of 15 matings) for 20% EY, (2) 46.5% (20 of 43 matings) for 5% EY, and (3) 58.5% (14 of 24 matings) for 20% EY + 60 mM β-CD. These data suggest that binding of cholesterol with β-CD enhances post-thaw fertility of jack semen samples. We conclude that acceptable pregnancy rates could be achieved with frozen–thawed jack semen samples cryopreserved in 5% EY or 20% EY + 60 mM β-CD using direct post-thaw insemination.

Keywords
doneky, seman, cryopreservation, cyclodestrin, cholestrol

Comments
This article is published as Jepsen, Rebecca J., Lawrence E. Evans, and Curtis R. Youngs. "Use of direct thaw insemination to establish pregnancies with frozen–thawed semen from a standard jack." *Journal of equine veterinary science* 30, no. 11 (2010): 651-656. doi:10.1016/j.jevs.2010.10.001.

CASE STUDY

Use of Direct Thaw Insemination to Establish Pregnancies with Frozen–Thawed Semen from a Standard Jack

Rebecca J. Jepsen, DVM, MS, PhD,[a] Lawrence E. Evans, DVM, PhD,[b] and Curtis R. Youngs, PhD[c]

ABSTRACT

Pregnancy rates reported after artificial insemination with frozen–thawed jack spermatozoa have been relatively low compared with those attained in other species. Cholesterol is known to influence post-thaw fertility of both jack and stallion semen, and altering the amount of cholesterol in the freezing extender may help improve the fertility of frozen–thawed jack semen samples. In this study, we report clinical work that was performed using semen samples collected from a single jack. Samples were extended in EZ Mixin OF and then slowly cooled to 5°C. Extended semen samples were centrifuged at 400 × *g* for 10 minutes and the supernatant was discarded. Spermatozoa were resuspended in freezing medium to a final concentration of 400 × 10^6 cells/mL and were later frozen in liquid nitrogen vapor. Freezing extender treatments containing 2% ethylene glycol included the following: (1) 20% egg yolk (EY), (2) 5% EY, and (3) 20% EY + 60 mM hydroxypropyl-β-cyclodextrin (β-CD). For this study, a total of 28 mares aged 2 to 18 years was used over five breeding seasons (82 total cycles). Mares were administered human chorionic gonadotropin to induce ovulation when the dominant follicle was ≥35 mm in diameter. They were inseminated within 6 hours before ovulation and again within 6 hours after ovulation. Pregnancy rates obtained were as follows: (1) 6.25% (one of 15 matings) for 20% EY, (2) 46.5% (20 of 43 matings) for 5% EY, and (3) 58.5% (14 of 24 matings) for 20% EY + 60 mM β-CD. These data suggest that binding of cholesterol with β-CD enhances post-thaw fertility of jack semen samples. We conclude that acceptable pregnancy rates could be achieved with frozen–thawed jack semen samples cryopreserved in 5% EY or 20% EY + 60 mM β-CD using direct post-thaw insemination.

Keywords: Donkey; Semen; Cryopreservation; Cyclodextrin; Cholesterol

INTRODUCTION

The birth of the first foal using frozen–thawed equine semen samples was reported in 1957 by researchers who used 10% glycerol (GLYC) in whole milk extender.[1] However, numerous reports exist which state that high concentrations of GLYC can be detrimental to the fertility of stallion semen.[2-5] There is a paucity of published data concerning establishment of pregnancies with cryopreserved jack semen samples when direct thaw and transfer methods of insemination are used.

In 1964, post-thaw motility as high as 70% was reported for jack semen samples that were frozen in 7.5% GLYC and 3.75% egg yolk (EY).[6] Three years later, one of the two mares inseminated with frozen–thawed jack semen samples that were cryopreserved in GLYC and thawed in sterile milk became pregnant.[7] More recently, semen samples from the endangered Poitou Jackass were cryopreserved in 4% GLYC supplemented with either 80 mM glutamine[8] or 10% quail EY instead of chicken EY.[9] No pregnancies were reported when jennies were artificially inseminated directly with these frozen–thawed jack semen samples[10]; however, a 62% pregnancy rate (eight of 13) was achieved when they were inseminated with semen samples that were extended post-thaw with skim milk extender, presumably diluting the GLYC.

Other cryoprotectants have been used with stallion semen in attempts to eliminate the use of GLYC and thereby avoid the reduction in post-thaw fertility.[2,11-17] Post-thaw motility of stallion semen samples cryopreserved in methyl formamide, dimethyl formamide, or ethylene glycol (EG) was found to be similar to that obtained with GLYC.[16] GLYC was deleterious to the fertility of both fresh and cryopreserved jack semen samples, but this reduced fertility was not observed when either dimethyl formamide or EG replaced GLYC in fresh extended jack semen samples.[18]

From the Animal Resource Unit, National Centers for Animal Health, Ames, IA[a]; Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA[b]; and Department of Animal Science, College of Agriculture and Life Sciences, Iowa State University, Ames, IA[c].
Reprint requests: Curtis R. Youngs, PhD, Department of Animal Science, Iowa State University, 2356B Kildee Hall, Ames, IA 50011.
0737-0806/$ - see front matter

doi:10.1016/j.jevs.2010.10.001

Cyclodextrins are water-soluble macrocyclic oligosaccharides that contain a hydrophobic center or pocket that can accommodate nonpolar substances.[15,19,20] β-cyclodextrins can readily accept cholesterol to form inclusion complexes because of the size of their pores (center).[15,20] Various forms of β-cyclodextrins have been added directly to the freezing extender for cryopreservation of boar semen.[21-23] Post-thaw acrosome integrity and motility were improved when either 2-hydroxypropyl-β-cyclodextrin (β-CD) or methyl-β-cyclodextrin was added to freezing extender during cooling. The protective effect of β-cyclodextrin may be because of cholesterol efflux from the sperm plasma membranes, which causes an increase in the fluidity of the cell membrane. This fluidity in turn imparts a resistance to cold shock during the cryopreservation process.[23] Alternatively, other researchers hypothesized that the increase in post-thaw viability of boar semen cryopreserved with 40 or 60 mM 2-β-CD was because cyclodextrin acted as a cholesterol shuttle to increase the sperm plasma membrane cholesterol:phospholipid ratio and thus reduce cold shock sensitivity of the sperm cells.[20] When 0.125 mM cyclodextrin–cholesterol complex was added to equine freezing extender, an increase in plasma membrane integrity was observed; however, this was accompanied by a noticeable decrease in pregnancy rates (75% vs. 25%).[24] Addition of cyclodextrin–cholesterol complex to bull sperm also improved post-thaw motility but not fertility.[13,25] Supplementation of freezing extender with methyl-β-cyclodextrin resulted in increased post-thaw motility and viability in jacks; however, fertility was not assessed.[26]

A client-owned standard jack was presented to the Iowa State University College of Veterinary Medicine for semen collection and semen freezing. The resultant frozen semen samples were prepared with the intention of inseminating the client-owned mares, several of which were draft mares. Concern for potential injury to the jack during natural mating with the draft mares was the primary impetus for the request to cryopreserve the semen. The objective of this clinical trial was to establish a freezing protocol for jack semen that would successfully result in pregnancies with a direct thaw and transfer method of insemination.

MATERIALS AND METHODS

The 5-year-old, 360-kg standard jack used in this study was known to be fertile, having sired several mule foals as a result of live coverage. Semen was collected from the jack with the aid of an ovariectomized, hormonally stimulated, and sedated mount mare who was restrained in palpation stocks. After the development of an erection during the teasing process, the jack's penis was gently washed with warm water and dried with a paper towel. The mount mare was removed from the stocks and paraded around the jack, who was tethered to a sturdy post, in order to stimulate another erection after the washing. Once an erection redeveloped, the mare was twitched, the jack was allowed to mount, and semen collection was performed with the use of a Missouri-type artificial vagina.

Cryopreservation Process

After the semen had been analyzed for motility, morphology, and concentration, it was extended in EZ Mixin OF (Animal Reproduction Systems, Chino, CA, USA) to a final concentration of approximately 50×10^6 cells per mL of extended semen. The extended semen was slowly cooled to 5°C in a water bath and then centrifuged in a refrigerated centrifuge at $400 \times g$ for 10 minutes. The supernatant was siphoned off and discarded. The remaining sperm cell pellet was resuspended in freezing extender to a final concentration of 400×10^6 cells per mL. Freezing extender treatments containing 2% EG included the following: (1) 20% EY, (2) 5% EY, and (3) 20% EY + 60 mM β-CD (refer to section on establishment of β-CD concentration in the following paragraphs). The resuspended sperm cells were packaged in 0.5-mL polyvinylchloride straws, equilibrated for 30 minutes, and then placed in liquid nitrogen vapor (−160°C) for a minimum of 20 minutes, at which time the straws were plunged into liquid nitrogen for storage until the time of analysis or insemination. For post-thaw analysis and artificial insemination, each straw of semen was removed from the liquid nitrogen storage tank and immediately placed in a water bath at 37°C for 30 seconds.

Establishment of β-CD Concentration

A small trial using the client-owned jack was conducted to establish an appropriate concentration of β-CD that can be used as a supplement in the freezing extender. The semen collection and processing procedures were as previously described. The following six different concentrations of β-CD were examined in the freezing medium (20% EY + 2% EG in EZ Mixin OF): 0 mM, 10 mM, 20 mM, 40 mM, 60 mM, and 80 mM. Eight ejaculates were collected, and each ejaculate was split and frozen in each of two or three different concentrations of β-CD in a balanced incomplete block design. Each treatment was replicated three times.

Post-thaw sperm viability was determined by flow cytometry using a dual-stain technique.[16,27,28] LIVE/DEAD Sperm Viability Kit (L-7011; SYBR-14 and propidium iodide [PI]) was purchased from Molecular Probes, Inc. (Eugene, OR, USA). HEPES, NaCl, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). A 1:50 dilution of SYBR-14 was prepared using HEPES-buffered saline solution (10 mM HEPES, 145 mM NaCl, 1% BSA; pH 7.4). The frozen semen samples were thawed as previously described and diluted 1:50 in pre-warmed HEPES buffer. A total of 5 μL of the diluted SYBR-14 were added to a 1 mL sample of

the diluted semen to achieve a final concentration of 100 nM SYBR-14. These samples were incubated for 5 minutes, and 5 μL of PI solution were added to the samples (final concentration of 12 μM PI). The samples were incubated for an additional 10 minutes before analyzing them with a Beckman Coulter XL-MCL flow cytometer (Beckman Coulter, Brea, CA, USA). The machine was set on a low flow rate, and excitation of the dyes was performed with a 488-nm argon laser. Fluorescence detector 1 (505–545 nm) detected the viable cells staining green with SYBR-14, whereas fluorescence detector 2 (605–635 nm) detected the dead cells staining red with PI. A total of 10,000 cell events was counted. Before the analysis of experimental samples, the protocol was verified by staining frozen–thawed samples with each of the dyes separately and then together. Additionally, unstained cells were also analyzed.

Motility characteristics of spermatozoa after thawing were analyzed using computer-assisted sperm analysis (CASA, Hobson SpermTracker version 7V2B, Hobson Vision Ltd., Baslow, Derbyshire, United Kingdom).

Inseminations

A total of 28 mares was used for fertility assessment over five breeding seasons. The mares were 2 to 18 years of age, ranged in weight from 454 to 818 kg, were mostly client-owned, and included seven Quarter horses, one American Paint horse, four Thoroughbreds, 11 Belgians, two Percherons, two Warmbloods, and one Standardbred. All mares were housed at Iowa State University College of Veterinary Medicine Teaching Hospital during the estrous period for the purpose of artificial insemination.

Mares were brought to the Teaching Hospital when they either displayed estrus or for an initial examination to determine the stage of the estrous cycle. Ovarian follicular assessment was made by rectal palpation and ultrasonography. Estrus was diagnosed when the mare met the following conditions: presence of a minimum of one follicle measuring >30 mm in diameter, ultrasonographic evidence of uterine edema, and estrous behavior (squatting, urinating, and general acceptance of a stallion). The mares were examined daily until the dominant follicle measured 35 mm or more in diameter, at which time assessments were made every 12 hours. Once the dominant follicle had attained a minimum diameter of 35 mm, 2,500 to 3,500 IU of Chorulon (Intervet, Millsboro, DE, USA) were administered intravenously to induce ovulation. Beginning at 24 hours after administration of Chorulon, ovarian status of mares was assessed every 6 hours. Mares were inseminated twice by the standard technique of uterine body deposition with approximately 300×10^6 motile sperm cells within 6 hours before and within 6 hours after ovulation. Semen was thawed as previously described. Pregnancy diagnosis was performed at 14 to 18 days after ovulation using transrectal ultrasonography.

Table 1. Post-thaw viability and motility of jack spermatozoa frozen in various concentrations of β-CD

Concentration of β-CD (mM)	Viability (%)[a] X ± SEM	Motility (%)[b] X ± SEM
0	27.3 ± 4.3	20.2 ± 5.8
10	28.3 ± 4.3	24.3 ± 5.8
20	27.2 ± 4.3	26.2 ± 5.8
40	31.5 ± 4.3	31.8 ± 5.8
60	43.5 ± 4.3	31.1 ± 5.8
80	37.1 ± 4.3	20.6 ± 5.8

β-CD, hydroxypropyl-β-cyclodextrin.
[a] Assessed through flow cytometry; $P < .11$.
[b] Determined through computer-assisted sperm analysis (CASA); $P > .60$.

Data Analysis

Fertility data were analyzed by χ^2 analysis. Viability and CASA motility from the β-CD dose response study were analyzed by analysis of variance using JMP software (SAS Institute, Inc. Cary, NC, USA).

RESULTS

Ejaculates were collected on multiple occasions over a 5-year period. Fresh ejaculates were evaluated for sperm morphology (75%–99% normal), pre-freeze motility (70%–90% motile), and volume (28–114 mL), all of which were considered within normal limits. A single straw from each freeze period was thawed and visually evaluated for post-thaw motility. When ejaculates were split into treatments, a single straw from each treatment was thawed and evaluated. Post-thaw progressive motility ranged from 10% to 30% and was not affected ($P > .10$) by freezing medium composition.

Post-thaw motility was not affected by the concentration of β-CD in the freezing extender ($P > .60$). However, a trend toward improved viability ($P < .11$) was observed with increasing concentration of β-CD (Table 1).

As shown in Table 2, pregnancy rate for freezing extenders containing 20% EY was lower ($P < .06$) compared with those containing 5% EY or 20% EY + 60 mM β-CD.

DISCUSSION

These data represent work that was completed in a clinical setting over a span of five breeding seasons. The fresh ejaculates were all acceptable for cryopreservation but did vary to some degree. The overall intent of the project was to achieve pregnancies for the client. Economics dictated that, when a particular freezing extender did not achieve acceptable pregnancy rates after six to eight matings, a new "direction" was sought (in contrast to what would

Table 2. Pregnancy data in mares inseminated with frozen–thawed jack semen

Freezing Treatment	Number of Inseminations	Number of Pregnancies	Pregnancy Rate (%)
20% EY	15	1	6.3[a]
5% EY	43	20	46.5[b]
20% EY + 60 mM β-CD	24	14	58.3[b]

EY, egg yolk; β-CD, hydroxypropyl-β-cyclodextrin.
[a,b] Means within a column with unlike superscripts are different ($P < .06$), χ^2 analysis.

occur in well-controlled nonclinical studies). For example, in the initial phase of this investigation, 4% GLYC was used as the cryoprotectant in the 20% EY treatment, but because no pregnancies were produced, focus was turned to the use of EG as the cryoprotective agent. As shown in Table 2, we were able to establish pregnancies using EG. It was previously reported that post-thaw motility of stallion semen was not different for freezing extenders containing either EG or GLYC as the cryoprotectant,[16] and further that fertility of fresh extended jack semen was not decreased when either dimethyl formamide or EG replaced GLYC as the cryoprotectant.[18] Our results are in agreement with those previous studies indicating that EG is an acceptable alternative to GLYC.

We achieved a pregnancy rate of 46.5% with freezing extenders that contained 5% EY when using a direct insemination method. French workers were unable to establish pregnancies in jennies who were inseminated, using a direct transfer method, with frozen–thawed Poitou Jackass semen that was cryopreserved with 10% quail EY and 4% GLYC.[6] However, they achieved a 62% pregnancy rate when the semen was diluted post-thaw with skim milk before insemination, suggesting that GLYC may interfere with post-thaw fertility. Another group of French researchers similarly reported a lack of pregnancy in jennies who were inseminated with Baudet du Poitou semen that was cryopreserved in 2.2% GLYC with 2% EY, and pregnancy occurred only when the frozen–thawed semen was washed in milk.[29] We were able to eliminate the step of post-thaw semen dilution/washing by using EG, a finding of great practical significance.

Post-thaw motility of cryopreserved jack semen is often considerably lower than that observed with fresh semen,[29-31] and pregnancy rates with frozen–thawed jack semen are also typically lower compared with those obtained with fresh semen.[29] The post-thaw motility reported in this study was ≤30% and was similar to that reported for donkey semen cryopreserved in 1.4% EG,[29] but the fact that we obtained pregnancy rates of 46.5% (5% EY) and 58.3% (20% EY + 60 mM β-CD) illustrates that post-thaw motility may not always be a strong predictor of pregnancy rate. Our results were comparatively better than those obtained when dimethylformamide was used in conjunction with GLYC to achieve a 40% pregnancy rate in mares inseminated with frozen–thawed jack semen.[32]

Post-thaw viability was not different for jack semen cryopreserved in varying concentrations of β-CD (Table 1). Although these data are from only a single jack (and inferences cannot be made for other jacks because of potential jack-specific effects[33]), a clear but nonsignificant trend toward improved viability was observed with increasing concentrations of β-CD, with the highest post-thaw viability occurring with the freezing extender containing 60 mM β-CD. Other researchers have noted an improvement in post-thaw viability of porcine semen when the freezing extender contained 40 or 60 mM β-CD.[20-23] Although statistical differences were not observed between varying concentrations of β-CD in our study, we felt that the trend in improvement, coupled with the data reported by others, was sufficient for us to proceed with inseminations using jack semen that had been cryopreserved with freezing extender containing 60 mM β-CD. The pregnancy rate was 58.3% for the 20% EY + β-CD treatment when a direct thaw and transfer insemination method was used, which was not different than that for the 5% EY group.

We believe that we are the first to report establishment of pregnancies with jack semen cryopreserved in the presence of β-CD. Data from our fertility trial suggest that the addition of β-CD sequestered enough cholesterol to decrease the inhibitory effect of high concentrations of EY (and presumably the high amount of cholesterol) on the post-thaw fertilizing capacity of jack semen. Our result is in agreement with a study in which ejaculates obtained from stallions with post-thaw motility above or below 40% that were treated with cholesterol-loaded cyclodextrins exhibited an increased proportion of intact sperm plasma membranes post-thaw.[34] Similarly, improvement in post-thaw plasma membrane integrity was observed in stallion semen cryopreserved with supplemental methyl-β-cyclodextrin-complexed cholesterol in 20% EY extender; however, this treatment led to a decrease in pregnancy rate.[24] In a different study, cholesterol addition enhanced stallion sperm plasma membrane integrity in frozen–thawed semen, but the use of methyl-β-cyclodextrin post-thaw to remove the added cholesterol was insufficient to subsequently enable adequate sperm acrosome reaction.[35] Denuded bovine oocytes inseminated in vitro with frozen–thawed jack semen resulted in fewer than 1.5 spermatozoa per oocyte,[36] suggesting that problems other than low rates of acrosome reaction (eg, DNA fragmentation[37]) contribute to the low

pregnancy rates previously observed with frozen–thawed jack semen.

These data indicate that acceptable pregnancy rates can be achieved with jack semen cryopreserved in a 2% EG freezing extender that contains 5% EY or 20% EY containing 60 mM β-CD when it is thawed and directly transferred into a mare. Further research is warranted to further elucidate the mechanism by which cholesterol influences fertility of jack semen. These findings may also have important implications for stallion semen cryopreservation.

ACKNOWLEDGMENTS

The authors thank Jerry and Janet King, owners of the jack, for their patience and support during the initial phases of this clinical project. Gratitude is also extended to Dr. Dave Guthrie for assistance with CASA. They also acknowledge gratefully the partial funding received from the USDA multi-state research project W-2171 "Germ Cell and Embryo Development and Manipulation for the Improvement of Livestock".

REFERENCES

1. Barker CAV, Gandier JCC. Pregnancy in a mare resulting from frozen epididymal spermatozoa. Can J Comp Med 1957;21:47–50.
2. Amann RP, Pickett BW. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. J Equine Vet Sci 1987; 7:145–173.
3. Crabo BG. Physiological aspects of stallion semen cryopreservation. In: Proceedings of the 47th American Association of Equine Practitioners; San Diego, CA; 2001:291–295.
4. Demick DS, Voss JL, Pickett BW. Effect of cooling, storage, glycerolization and spermatozoal numbers on equine fertility. J Anim Sci 1976;43:633–637.
5. Pace MM, Sullivan JJ. Effect of timing of insemination, numbers of spermatozoa and extender components on the pregnancy rate in mares inseminated with frozen stallion semen. J Reprod Fertil Suppl 1975;23:115–121.
6. Polge C, Minotakis, C. Deep freezing of jackass and stallion semen. In: Proceedings of the 5th International Congress on Animal Reproduction; Trento, Italy; 1964:545–552.
7. Krause D, Grove D. Deep-freezing of jackass and stallion semen in concentrated pellet form. J Reprod Fertil 1967;14:139–141.
8. Trimeche A, Renard P, Le Lannou D, Barriere P, Tainturier D. Improvement of motility of post-thaw Poitou jackass sperm using glutamine. Theriogenology 1996;45:1015–1027.
9. Trimeche A, Anton M, Renard P, Gandemer G, Tainturier D. Quail egg yolk: a novel cryoprotectant for the freeze preservation of Poitou Jackass sperm. Cryobiology 1997;34:385–393.
10. Trimeche A, Renard P, Tainturier D. A procedure for Poitou jackass sperm cryopreservation. Theriogenology 1998;50:793–806.
11. Chenier T, Merkies K, Leibo S, Plante C, Johnson W. Evaluation of cryoprotective agents for use in the cryopreservation of equine spermatozoa. In: Proceedings of Annual Meeting of the Society for Theriogenology; Baltimore, MD; 1998:52–53.
12. Gomes GM, Jacob JCF, Medeiros ASL, Papa FO, Alvarenga MA. Improvement of stallion spermatozoa preservation with alternative cryoprotectants for the Mangalarga Marchador breed. Theriogenology 2002;58:277–279.
13. Graham JK. An update on semen extenders and cryoprotectants. In: Proceedings of the 17th Technical Conference on Artificial Insemination and Reproduction; Middleton, WI; 1998:32–36.
14. Henry M, Snoeck PPN, Cottorello SCP. Post-thaw spermatozoa plasma membrane integrity and motility of stallion semen frozen with different cryoprotectants. Theriogenology 2002;58:245–248.
15. Mantovani R, Rota A, Faloma ME, Bailoni L, Vincenti L. Comparison between glycerol and ethylene glycol for the cryopreservation of equine spermatozoa: semen quality assessment with standard analyses and with the hypoosmotic swelling test. Reprod Nutr Dev 2002;42: 217–226.
16. Squires EL, Keith SL, Graham JK. Evaluation of alternative cryoprotectants for preserving stallion spermatozoa. Theriogenology 2004; 62:1056–1065.
17. Vidament M, Daire C, Yvon JM, Doligez P, Bruneau B, Magistrini M, et al. Motility and fertility of stallion semen frozen with glycerol and/or dimethyl formamide. Theriogenology 2002; 58:249–251.
18. Vincent P, Yvon JM, Martin FX, Bruneau B, Cinqualbre V, Berland C, et al. Le glycerol est-il un facteur limitant pour la conservation de la semence dans les espedes asine et equine? Journee de la Recherche Equine 2004;30:55–62.
19. Belmonte SA, Lopez CI, Roggero CM, De Blas GA, Tomes CN, Mayorga LS. Cholesterol content regulates acrosomal exocytosis by enhancing Rab3A plasma membrane association. Dev Biol 2005; 285:393–408.
20. Cross NL. Effect of methyl-B-cyclodextrin on the acrosomal responsiveness of human sperm. Mol Reprod Dev 1999;53:92–98.
21. Zeng WX, Terada T. Freezability of boar spermatozoa is improved by exposure to 2-hydroxypropyl-beta-cyclodextrin. Reprod Fertil Dev 2000;12:223–228.
22. Zeng WX, Terada T. Effects of methyl-beta-cyclodextrin on cryosurvival of boar spermatozoa. J Androl 2001;22:111–118.
23. Zeng WX, Terada T. Protection of boar spermatozoa from cold shock damage by 2-hydroxypropyl-beta-cyclodextrin. Theriogenology 2001;55:615–627.
24. Zahn FS, Papa FO, Dell'Aqua JA Jr. Cholesterol incorporation on equine sperm membrane: effects on post-thaw sperm parameters and fertility. Theriogenology 2002;58:237–240.
25. Medeiros CMO, Forell F, Oliveira ATD, Rodrigues JL. Current status of sperm cryopreservation: why isn't it better? Theriogenology 2002;57:327–344.
26. Álvarez AL, Serres C, Torres P, Crespo F, Mateos E, Gómez-Cuétara. Effect of cholesterol-loaded cyclodextrin on the cryopreservation of donkey spermatozoa. Anim Reprod Sci 2006;94:89–91.
27. Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995;53:276–284.

28. Garner DL, Johnson LA, Yue ST, Roth BL, Haugland RP. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. J Androl 1994;15:620–629.
29. Vidament M, Vincent P, Martin F-X, Magistrini M, Blesbois E. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not. Anim Reprod Sci 2009;112:22–35.
30. Flores E, Taberner E, Rivera MM, Peña A, Rigau T, Miró J, et al. Effects of freezing/thawing on motile sperm subpopulations of boar and donkey ejaculates. Theriogenology 2008;70:936–945.
31. Canisso IF, Carvalho GR, Morel MD, Ker PG, Rodrigues AL, Silva EC, et al. Seminal parameters and field fertility of cryopreserved donkey jack semen after insemination of horse mares. Equine Vet J (in press). doi: 10.1111/j.2042-3306.2010.00130.x.
32. Oliveira JV, Alvarenga MA, Melo CM, Macedo LM, Dell'Aqua JA Jr, Papa FO. Effect of cryoprotectant on donkey semen freezability and fertility. Anim Reprod Sci 2006;94:82–84.
33. Rota A, Magelli C, Panzani D, Camillo F. Effect of extender, centrifugation and removal of seminal plasma on cooled-preserved Amiata donkey spermatozoa. Theriogenology 2008;69:176–185.
34. Moore AI, Squires EL, Graham JK. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology 2005; 51:241–249.
35. Oliveira CH, Vasconcelos AB, Souza FA, Martins-Filho OA, Silva MX, Varago FC, et al. Cholesterol addition protects membrane intactness during cryopreservation of stallion sperm. Anim Reprod Sci 2010;118:194–200.
36. Taberner E, Morató R, Mogas T, Miró J. Ability of Catalonian donkey sperm to penetrate zona pellucida-free bovine oocytes matured in vitro. Anim Reprod Sci 2010;118:354–361.
37. Cortés-Gutiérrez EI, Crespo F, Gosálvez A, Dávila-Rodríguez MI, López-Fernández C, Gósalvez J. DNA fragmentation in frozen sperm of *Equus asinus*: Zamorano-Leonés, a breed at risk of extinction. Theriogenology 2008;69:1022–1032.