

A pilot study: Comparison of pregnancy rates from vitrified Jersey embryos utilizing direct transfer methods with pregnancy rates of Jersey and Holstein embryos frozen in ethylene glycol or glycerol by slow-cooling methods

Richard G. Steel

Evergreen Veterinary Reproductive Services, Tillamook, OR

Abstract

It has been previously reported that pregnancy rates from frozen Jersey embryos are lower than pregnancy rates from other breeds of cattle. This study incorporated 1.5M ethylene glycol or 1.4M glycerol as cryoprotectants and utilized slow-cooling freeze programs. The present study was undertaken to determine if the vitrification of Jersey embryos in 0.25 cc straws could result in a more favorable pregnancy rate than previously reported. In addition, an in-straw dilution of cryoprotectants with resulting direct transfer (DT) of embryos was evaluated to determine if this methodology is compatible with vitrification processes. Ten superovulations of nine Jersey donor cows yielded 50 embryos that were vitrified and subsequently thawed and transferred into suitable recipients using DT methodology. The resulting pregnancy rate (28%) is numerically lower than retrospective pregnancy rates achieved from Jersey embryos slow-cooled in 1.4M glycerol or 1.5M ethylene glycol (46.2% and 39.9% respectively). However, two Jersey donors achieved reasonable pregnancy rates with their vitrified embryos (50%). This study has shown that average pregnancy rates of vitrified Jersey embryos utilizing DT methodology is numerically lower than average pregnancy rates of conventional, slow-cooled embryos. However, due to the wide range of pregnancy rates in this study, individual donor cows may have different cryotolerances to this vitrification method. Further modifications of vitrification systems utilizing DT methods may eventually improve overall pregnancy rates of frozen Jersey embryos.

Keywords: Vitrification, pregnancy rates, direct transfer, slow-cooling, Jersey embryos

Introduction

Cryopreservation of bovine embryos has been a routine procedure performed in commercial embryo transfer businesses for many years. Pregnancy rates resulting from the transfer of frozen bovine embryos are for the most part very predictable as evidenced by the widespread domestic and international commercial activity of selling frozen embryos. Many types of biologic specimens have been successfully cryopreserved; however, there have been some exceptions related to a decreased tolerance to chilling. Some notable examples are porcine embryos,¹ oocytes,² in-vitro produced embryos³ and *Bos indicus* embryos.⁴ Several reports in the literature have described lower pregnancy rates of frozen Jersey embryos when compared to pregnancy rates of frozen embryos from other dairy breeds.⁵⁻⁷ There has been some speculation about the reasons for this occurrence, especially with the discovery that Jersey embryos may have a relatively higher lipid content than embryos from other breeds.^{7,8} Some investigators have observed that embryos with higher lipid concentrations experience depressed frozen embryo pregnancy rates.^{1,7,9,10} This has led the industry to test other types of cryoprotectants and procedures that may be more appropriate for Jersey embryos.

Vitrification, although not a new process,¹¹ is a freezing procedure that has been successfully applied to many types of specimens.¹²⁻²⁰ Recently, studies have compared pregnancy rates from embryos frozen by slow-cool conventional methods with embryos frozen by vitrification.²¹⁻³⁰ Some of these studies have shown vitrification to be superior to conventional freezing techniques.^{22,27,31-33}

Most vitrified specimens undergo a serial dilution of cryoprotectants in a laboratory setting prior to culture and/or transfer. In-straw dilution of cryoprotectants was described a number of years ago³⁴ and has the advantage of bypassing any laboratory environment by allowing DT of an embryo into a recipient animal after thawing and equilibration. However, with the advent of 1.5M ethylene glycol as a cryoprotectant in slow-cooling systems, serial dilution of cryoprotectants is not necessary, making use of this cryoprotectant and DT the method of choice in the embryo transfer industry. Ninety percent of all bovine embryos thawed in the US in 2007 utilized the DT method.³⁵

To date, there have not been sufficient data generated to determine if vitrification of Jersey embryos would increase frozen embryo pregnancy rates. There have also been very few studies involving in-straw dilution of cryoprotectants of vitrified embryos with subsequent DT.^{26,36,37} Thus, the aim of this study was to compare pregnancy rates of vitrified Jersey embryos utilizing in-straw dilution of cryoprotectants and direct DT with retrospective pregnancy rates of Jersey and Holstein embryos frozen in glycerol or ethylene glycol by conventional slow-cooling methods.

Materials and methods

Animals

Ten superovulations were performed on nine lactating Jersey donor cows at least two years of age on five different dairies. Donors were examined for normal reproductive health before enrollment. One donor cow was superovulated twice. Recipient animals were both lactating cows and virgin heifers and included Jersey and Holstein breeds.

Superovulation

All donors were treated with a progesterone-containing vaginal insert (Eazi-Breed™ CIDR®, Pfizer Animal Health, New York, NY) at random stages of the estrous cycle four days prior to superstimulation. Donors also received 100 mcg gonadotropin-releasing hormone (GnRH, Cystorelin®, Merial, Duluth, GA) 60 hours prior to superstimulation. Superstimulation was accomplished with a decreasing dose of follicle stimulating hormone (FSH; Folltropin®-V, Bioniche, Bogart, GA) twice daily over four days for a total dose of 300 mg. Thirty-seven mg and 25 mg dinoprost tromethamine (Lutalyse®, Pfizer Animal Health) was given at the same time as the fifth and sixth FSH injection, respectively. Vaginal inserts were removed when the sixth FSH injection was given. Donor cows were inseminated twice with one unit of semen at 12 and 24 hours after the onset of estrus. Embryos were collected 7.5 days after the onset of estrus.

Recipient synchronization

Recipients were synchronized with either one or two injections of 25 mg dinoprost tromethamine or a progesterone-containing vaginal insert. Recipients synchronized with a single injection of dinoprost tromethamine were observed for estrus two to five days after treatment. Recipients synchronized with two injections were treated at an interval of 12-14 days and observed for estrus two to five days after the second injection. Progesterone-containing vaginal inserts were placed at random stages of the estrous cycle and left in place for seven days. Twenty-four hours prior to insert removal, recipients were injected with 25 mg dinoprost tromethamine. The animals were observed for estrus for five days after removal of the inserts. Only recipients detected in standing estrus and that had a mature corpus luteum on the day of transfer were used.

Embryos frozen conventionally

Retrospective data from the last 15 years were compiled to compare pregnancy rates utilizing slow-cooling protocols. Grade 1 or 2 Jersey and Holstein embryos were frozen by conventional slow-cooling methods with either 1.4M glycerol (GLY) or 1.5M ethylene glycol (EG). All embryos were thawed for six seconds in air, then 15 seconds in 30 °C water. Embryos frozen in glycerol were passed through a three step serial dilution in the presence of 0.5M sucrose. After serial dilution, embryos were transferred within one hour of thawing. Embryos frozen in ethylene glycol were immediately loaded into a transfer gun after thawing and transferred within 10 minutes.

Embryos vitrified

An aluminum block, partially submerged in a liquid nitrogen bath, was utilized as a vitrification chamber. This device contains holes 123 mm deep with a diameter that allows a 0.25 cc straw to fit snugly against the metal sidewall with as little air interface as possible. Grade 1 and 2 embryos were first

exposed to vitrification solution 1 (5.0M ethylene glycol) for three minutes then placed in a 15 ul drop of vitrification solution 2 (7.0M ethylene glycol plus 0.5M galactose with 18% Ficoll™ [GE Healthcare, Piscataway, NJ]). Embryos were immediately loaded into a 0.25 cc straw in the following manner: 173 ul dilution solution (1.0M galactose), a 10 mm air bubble, embryo in a 15 ul micro drop of vitrification solution 2, a 10 mm air bubble, with the remaining space filled with diluent solution. Straws were then sealed and embryos were visualized to confirm their exact location prior to plunging. Embryos were exposed to vitrification solution 2 for 45 seconds after which the straw was inserted into the vitrification device for 60 seconds. Straws were then immediately plunged into liquid nitrogen and stored in goblets until transfer between one hour and 30 days later. Embryos were thawed six seconds in air, then 20 seconds in 35 °C water. Straws were dried and shaken in a downward manner until all media within the straw was mixed. Immediately after thawing, embryos were visually located during a four minute equilibration at 25 °C to ensure they were not in the end of the straw that was to be cut. Embryos were then loaded into a transfer gun and transferred into a suitable recipient within ten minutes after equilibration.

Pregnancy examination

All animals were manually examined for pregnancy 35 to 60 days after the calculated date of conception.

Results

A total of 52 grade 1 and 2 embryos were recovered from ten superovulations of nine Jersey donor cows for an average of 5.2 embryos per flush (Table 1). Fifty embryos were vitrified and two embryos were discarded during the vitrifying procedure. This was due to the fact that these two embryos were exposed to vitrification solution 2 longer than 45 seconds due to technical difficulties and were excluded from this study. A total of 50 vitrified Jersey embryos were thawed and transferred into suitable recipients resulting in 14 pregnancies (28%). Pregnancy rates for embryos frozen from each individual donor cow ranged from 0 to 50% (Table 1). Pregnancy rates on individual dairies ranged from 0 to 41.7% (Table 2). Upon ultrasonic examination at 75 days gestation, one recipient on Farm 2 was found to have twin female fetuses, the result of spontaneous splitting of a vitrified embryo after thawing.

Discussion

An average pregnancy rate of 28% achieved from the transfer of 50 vitrified Jersey embryos is numerically lower than the average pregnancy rates achieved from the transfer of Jersey and Holstein embryos slow-cooled in 1.4M glycerol or 1.5M ethylene glycol (Table 3). Pregnancy rates of Jersey embryos, regardless of freezing technique, were numerically lower than pregnancy rates of frozen Holstein embryos. Although data are limited, vitrified embryos from two donors resulted in pregnancy rates of 50.0%. A wide variation of pregnancy rates (0-50.0%) from individual Jersey donors is apparent in this study of vitrified embryos, and, in the author's experience, Jersey embryos frozen by conventional slow-cooling methods.* The variation between Jersey donor cows that achieve above average pregnancy rates with frozen embryos, and those that underachieve on a consistent basis, indicates there may be differences in freezing tolerances among individual donors within the same breed. This pilot study did not confirm the results of a previous study in which bovine embryos were vitrified in 6.5M glycerol and cryoprotectants diluted after thawing in 1M sucrose prior to DT. Utilizing 728 recipients, pregnancy rates from vitrified embryos were similar to those from embryos slow-cooled and frozen in 1.5M glycerol with a three step serial dilution after thawing (45.1% vs. 44.5%, respectively).³⁶ With the methods used in the current study, larger data sets would be necessary to test for significant differences in pregnancy rates between vitrified Jersey embryos and Jersey embryos frozen with conventional slow-cooling methods.

It is important to note that proper placement of the embryo within the vitrifying environment is extremely important when making critical evaluations of this process. Prior to this study, 50 unfertilized

* R. Steele, Unpublished observations

bovine ova were vitrified and it was noted that two ova were improperly located within the diluent solution after the straws were sealed. In the current study, all embryos were visually located prior to freezing to confirm their proper location within the vitrification environment. In addition, upon thawing and mixing of diluent within the straw, embryos were located and tracked during equilibration to ensure they did not drift too close to the sealed end that was to be opened prior to transfer.

Conclusion

Vitrification of mammalian embryos has been shown to be successful in a variety of clinical settings. Perhaps the greatest challenge in undertaking this study was to incorporate an in-straw dilution and subsequent direct transfer method of vitrified embryos. Can acceptable pregnancy rates be achieved with vitrified embryos without the time and expense of passing embryos through a serial dilution of cryoprotectants? Can proper vitrification occur within the confines of a 0.25 cc straw where a large sample size (15 ul micro drop) and the insulating effects of the straw may be detrimental to the process? Both of these aspects would have great bearing on achieving an ultra-rapid cooling rate, a factor of critical importance in the vitrification process. This study has shown that average pregnancy rates from vitrified Jersey embryos are numerically lower than the average pregnancy rates of Jersey embryos slow-cooled by conventional methods. However, some individual exceptions were noted as vitrified embryos from two Jersey donors achieved a 50% pregnancy rate. Donors that achieve high average pregnancy rates often demonstrate the potential of a new methodology. Although Jersey embryos may possess unique characteristics in their cryotolerance, modification of current vitrification processes utilizing DT methods, combined with further field trials with larger numbers of animals, may help improve overall pregnancy rates with frozen Jersey embryos.

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Table 1. Summary table of superovulation and vitrification results.

Superovulation Number	Total Recovered	Total Grade 1 & 2	Total Grade 3	Total Degen.	Total Unfert.	Total Vitrified	Total Thawed	Total Preg.	Preg Rate (%)
1	15	3	0	0	12	3	3	0	0.0
2	15	12	1	0	2	11 [†]	11	2	18.2
3	7	5	0	1	1	5	5	2	40.0
4	5	0	0	0	5	0	N/A	N/A	N/A
5	6	3	0	0	3	2 [†]	2	0	0.0
6	13	10	0	2	1	10	10	5	50.0
7	6	6	0	0	0	6	6	1	16.7
8	22	8	0	0	14	8	8	4	50.0
9	3	0	0	2	1	0	N/A	N/A	N/A
10	7	5	1	0	1	5	5	0	0.0
Totals	99	52	2	5	40	50	50	14	28.0
Averages	9.90	5.20	0.20	0.50	4.00	5.00			

[†]One embryo discarded due to improper timing of vitrification process.

Table 2. Summary of pregnancies obtained at five different Jersey farms.

Farm	Total pregnancies	Total transferred	Pregnancy rate (%)
1	0	5	0.0
2	7	25	28.0
3	5	12	41.7
4	2	5	40.0
5	0	3	0.0
Totals	14	50	28.0

Table 3. Pregnancy rates resulting from the transfer of fresh and frozen Holstein and Jersey embryos.

Embryos	Holstein Embryos		Jersey Embryos	
	No. Transferred	% Pregnant	No. Transferred	% Pregnant
Fresh [‡]	3376	65.3	1161	62.8
Frozen GLY [‡]	836	57.7	593	46.2
Frozen EG [‡]	1111	53.8	193	39.9
Vitrified	---	---	50	28.0

[‡]Historical data⁵

References

- Berthelot F, Martinat-Botte F, Vajta G: Cryopreservation of porcine embryos: state of the art. *Livest Prod Sci* 2003;83:73-83.
- Otoi T, Kamamoto K, Koyama N, et al: Cryopreservation of mature bovine oocytes following centrifugation treatment. *Cryobiology* 1997;34:36-41.
- Massip A, Mermillod P, Dinnyes A: Morphology and biochemistry of in-vitro produced bovine embryos, implications for their cryopreservation. *Hum Reprod*;1995;10:3004-3011.
- Visintin JA, Martins JFP, Bevilacqua EV, et al: Cryopreservation of *Bos taurus* vs *Bos indicus* embryos: are they really different? *Theriogenology* 2002;57:345-359.
- Steel R, Hasler JF: Pregnancy rates resulting from the transfer of fresh and frozen Holstein and Jersey embryos. *Reprod Fertil Dev* 2004;16:182-183.
- Kieler M: Embryo comparisons for five dairy breeds. *Proc Am Embryo Transf Assoc*; 2008. p. 49-50.
- Weathers J: Early indications of breed differences for cryopreservation of embryos in cattle [thesis]. Lubbock (TX): Texas Tech University; 2008.
- Pegoraro LM, Barros S, Sinowatz F, et al: Lipidic content in Jersey blastocysts compared with Holstein and IVP embryos. *Reprod Fertil Dev* 2004;16:268.
- George F, Daniaux C, Genicot G, et al: Lipid content, resistance to cryopreservation and sex ratio of in-vitro bovine blastocysts produced in a serum free media. *Reprod Fertil Dev* 2006;18:188-189
- Abe H, Yamashita S, Satoh T, et al: Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Mol Reprod Dev* 2002;61:57-66.
- Vafta G, Nagy ZP: Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reprod Biomed Online* 2006;12:779-796.
- Siedel G: New methods of cryopreserving bovine embryos. *Proc Am Embryo Transf Assoc* 2007. p. 98-101.
- Ishimore H, Saeki K, Inai M, et al: Vitrification of bovine embryos in a mixture of ethylene glycol and dimethyl sulfoxide. *Theriogenology* 1993;40:427-433.
- Stachecki J, Garrisi J, Sabino S, et al: A new safe, simple and successful vitrification method for bovine and human blastocysts. *Reprod Biomed Online* 2008;17:360-367.
- Vajta G: Vitrification of the oocytes and embryos of domestic animals. *Anim Reprod Sci* 2000;60-61:357-364.
- Campos-Chillos LF, Suh TK, Barelo-Fimbres M, et al: Vitrification of early stage bovine and equine embryos. *Theriogenology* 2009;71:349-354.
- Kuwayama M, Vajta G, Kato O, et al: Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online* 2005;11:300-308.
- Frers L, Hepburn J, Mandriaza-Munoz J, et al: Vitrification of bovine in-vitro produced and in-vivo embryos. *Reprod Fertil Dev* 2009;21:131-132.
- Vanderzwalmen P, Zech N, Greindl AJ, et al: Cryopreservation of human embryos by vitrification. *Gynecol Obstet Fertil* 2006;34:760-769.

20. Chun-Chia H, Tsung-Hsien L, Shee-Uan C, et al: Successful pregnancy following blastocysts cryopreservation using super-cooling ultra rapid vitrification. *Hum Reprod* 2005;20:122-128.
21. da Silva FM, Metelo R: Relation between physical properties of the zona pellucida and viability of bovine embryos after slow-freezing and vitrification. *Reprod Domest Anim* 2005;40:205-209.
22. Stehlik E, Stehlik J, Datayama KP, et al: Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. *Reprod Biomed Online* 2005;11:53-57.
23. Vajta G, Kuwayama M: Improving cryopreservation systems. *Theriogenology* 2006;65:236-244.
24. Dupras R, Dupras J, Chorfi Y: Cell integrity of Holstein and Jersey embryos. Comparison of two freezing methods. *Reprod Fertil Dev* 2008;20:149-150.
25. Liebermann J, Tucker MJ: Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertil Steril* 2006;86:20-26.
26. Massip A, Vanderzwalmen P, Ectors F: Recent progress in cryopreservation of cattle embryos. *Theriogenology* 1987;27:69.
27. Loutradi, K, Kolibianakis EM, Venetis CA, et al: Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. *Fertil Steril* 2008;90:186-193.
28. Massip A: Cryopreservation of embryos of farm animals. *Reprod Domest Anim* 2001;36:49-55.
29. Liebermann J, Dietl J, Vanderzwalmen P, et al: Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now? *Reprod Biomed Online* 2003;7:623-633.
30. Kolibianakis EM, Venetis CA, Tariatzis, BC: Cryopreservation of human embryos by vitrification or slow freezing: which one is better? *Curr Opin Obstet Gynecol* 2009;21:270-274.
31. Kuleshova L, Lopata A: Vitrification can be more favorable than slow cooling. *Fertil Steril* 2002;78:449-454.
32. Al-Hasani S, Ozmen B, Koutlaki N, et al: Three years of routine vitrification of human zygotes: is it still fair to advocate slow-rate freezing? *Reprod Biomed Online* 2007;14:288-293.
33. Urman B: Vitrification of human embryos is superior to conventional slow freezing (review). *Fertility Weekly* 2008 Nov 10.
34. Leibo S: A one step method for direct nonsurgical transfer of frozen-thawed bovine embryos. *Theriogenology* 1984;21:767-790.
35. American Embryo Transfer Association: Statistical Report 2007. Champaign (IL): AETA.
36. Van Wagendonk-de Leeuw AM, den Daas JHG, Rall WF: Field trial to compare pregnancy rates of bovine embryo cryopreservation methods. *Theriogenology* 1997;48:1071-1084.
37. Campos-Chillon LF, Walker DJ, de la Torre-Sanchez JF, et al: In vitro assessment of a direct transfer vitrification procedure for bovine embryos. *Theriogenology* 2006;65:1200-1214.