THESIS

VITRIFICATION OF IN VITRO- AND IN VIVO-PRODUCED BOVINE EMBRYOS FOR DIRECT TRANSFER

Submitted by

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ABSTRACT

VITRIFICATION OF IN VITRO- AND IN VIVO-PRODUCED BOVINE EMBRYOS FOR DIRECT TRANSFER

The overall objective of my thesis research was to improve procedures for vitrifying bovine blastocysts so as to enable direct embryo transfer to the uterus. Blastocysts were produced using standard in vitro procedures in Experiments 1, 2, and 3. Procedures were done at room temperature, 22 ± 2 °C. Unless otherwise mentioned, all media were made in SynGro®. In Experiment 1, base media contained either 1) normal concentrations of sodium (120 mM) and calcium (2 mM);(CON) or 2) 60 mM sodium + 60 mM choline chloride and 0.5 mM calcium (LOW). Blastocysts were exposed to 5 M ethylene glycol (V1) for 3 min and moved to 6.5 M ethylene glycol + 0.5 M galactose + 18% Ficoll (V2). Straws (0.25 mL) were loaded with a column of 120 µl 1 M galactose followed by an air bubble, then V2 containing embryos followed by an air bubble, and 60 µl 1 M galactose followed by sealing with a plastic plug. After 35 s, embryos were vitrified by either 1) standard cooling in liquid nitrogen cooled air (AIR) for 1 min or 2) cooling via contact of straw walls with columns drilled into an aluminum block immersed in liquid nitrogen (BLK) for 2 min and then directly plunged into liquid nitrogen. These combinations resulted in 4 treatments (AIR x CON; n = 61, AIR x LOW; n = 58, BLK x CON; n = 73, BLK x LOW; n = 54). BLK Embryos were warmed by holding straws in air for 10 s, placing them in a water bath at 37 °C for 20 s, mixing embryos with galactose diluent in the straw for 2 min and

expelling. Embryos were recovered, rinsed through holding medium, and cultured in chemically defined medium (similar to synthetic oviduct fluid (SOF)) for 24 h before being evaluated for survival. Post warming survival did not differ ($P > 0.10$) between treatments (AIR x CON = 42.0%; AIR x LOW = 26.8%; BLK x CON = 21.8%, BLK x LOW = 24.5%). Despite lack of statistical significance, we recommend use of LOW base media because both sodium and calcium levels are reduced. Use of this media should therefore have less chance of sodium and calcium toxicity, and could deter apoptosis. The BLK vitrification method is both easier to use and more consistent.

In Experiment 2, we sought to identify the most efficacious cryopreservation method for in vitro-produced bovine blastocysts that would enable direct embryo transfer from 0.25 mL straws used as containers for cryopreservation. Although not a method for direct transfer, Cryotops were chosen to serve as positive controls (CON), as they are the industry standard for vitrification of human embryos. Embryos were cryopreserved by vitrification with a Cryotop (CON; n = 118), using an aluminum block (BLK; n = 128), or by slow freezing (SLF; n = 131). Vitrification procedures were as described above for BLK with the exception that CON embryos were placed in < 1 µl V2 onto Cryotops, and after 35 s, vitrified by plunging directly into liquid nitrogen. Embryos cryopreserved via SLF were exposed to 1.36 M glycerol in modified Dulbecco's PBS + 0.4% BSA (PBS) for 10 min, loaded into 0.25 mL straws, and placed into a freezing machine. Straws were cooled to -6 °C at 4 °C per min, held at -6 °C for 5 min, seeded, held at -6 °C for an additional 10 min, and then

cooled to -30 °C at 0.5 °C per min and plunged into liquid nitrogen. After storage for at least 24 h in liquid nitrogen, embryos were warmed/thawed. Embryos cryopreserved via CON were removed from Cryotops by direct placement into a 200 µl drop of 1 M galactose for 2 min, whereas BLK/SLF embryos were warmed/thawed as described above with the exception that glycerol was removed in three 6 min steps from SLF embryos: 0.8 M glycerol + 0.3 M sucrose; 0.4 M glycerol + 0.3 M sucrose; and 0.3 M sucrose followed by PBS for 2 min. After recovery, embryos were rinsed through holding medium and cultured as described above. Post warming survival was greater ($P < 0.01$) for CON than BLK (85.9% and 70.6%, respectively); BLK was greater ($P < 0.01$) than SLF (56.1%). Although BLK resulted in lower post-warming survival than CON, it may be an acceptable method for direct transfer, which yielded greater post-warming survival than SLF, the current method used for cryopreservation of bovine embryos.

In Experiments 3 and 4, the objective was to compare pregnancy rates of recipients of in vitro-(Exp 3) or in vivo-produced bovine blastocysts (Exp 4) cryopreserved via VIT versus SLF. In vitro-produced embryos were produced by standard procedures. In vivo-produced embryos were recovered 7 d post estrus from crossbred, nonlactating superovulated beef cows. Embryos were cryopreserved via BLK vitrification (VIT; Exp 3, n = 78; Exp 4, n = 46) or slow freezing (SLF; Exp 3, n = 78; Exp 4, n = 44). Embryos were cryopreserved and warmed/thawed as described above followed by nonsurgical transfer into non-pregnant cows culled for unknown reasons, but with normal-appearing

reproductive tracts. Recipients were d 7 ± 0.5 of the estrous cycle, and each received 2 embryos into the uterine horn ipsilateral to the corpus luteum. Pregnancy diagnosis was performed at d 37 ± 2 via ultrasonography. Survival rate per embryo (normal fetus with heartbeat) did not differ ($P > 0.10$) between methods (Exp 3, VIT = 14.1%; SLF = 16.7%; 9 of 15 pregnant cows carried twins; Exp 4, VIT = 45.7%; SLF = 38.6%; 17 of the 21 pregnant cows carried twins). Therefore, VIT was similarly efficacious to SLF for cryopreservation of bovine embryos, and simpler, requiring less equipment, time, and expense.

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Chapter 1

Introduction

Over 1.1 million embryos were transferred in 2009, made easier by the assistance of new technologies. Superovulation is evolving by way of a slow release formula only requiring a single injection as opposed to the conventional 8 injection method. Transvaginal follicular aspiration of oocytes has become more common (13.4% increase from 2008 to 2009; American Embryo Transfer Association, 2010) requiring in vitro-fertilization and culture of embryos. In vitro-production of bovine embryos worldwide has increased 12.7% to 379,000 transferrable embryos produced in 2009, but very few were cryopreserved (Stroud, 2010). Improvements in maturation, fertilization, culture, and cryopreservation of in vitro-produced embryos are having a significant impact on commercial embryo transfer. This project focuses on advancing knowledge of cryopreservation, specifically vitrification.

Vitrification is the solidification of a solution brought about not by crystallization, but by extreme elevation in viscosity during cooling (Fahy, 1984). Cryopreservation without forming damaging ice crystals is the foremost advantage of vitrification over conventional freezing, but it also offers the benefits of minimizing time and cost associated with the technique. Although hundreds of

vitrification studies have been published (Vajta and Nagy, 2006) there has been little application of the technique in commercial bovine embryo transfer (< 0.3%; American Embryo Transfer Association, 2010). We sought to improve vitrification media and simplify the cooling method while enabling direct transfer with the following experiments. Also, we compared pregnancy rates of recipients receiving vitrified versus conventionally frozen embryos.

Chapter 2

Review of Literature

Cryopreservation Basics

The ultimate goal of cryopreservation of cells is to cool them to a point at which intracellular functions stop, and therefore cells do not progress in development or require additional energy inputs. Cells must also maintain their physical and chemical integrity, which will allow them to function upon warming. Both thermal shock and ice formation are barriers to successful cryopreservation, but these obstacles can be eliminated by using cryoprotective agents, controlling cooling rates, and exposing cells to a high salt medium, ultimately dehydrating them. Excessive dehydration of cells has its own set of problems, including permanent damage to cellular structures, pH changes leading to protein damage, and concentration of ions resulting in toxicity. In fact, all protocols associated with freezing and vitrification depend on a number of factors, many of which conflict with one another and are not entirely understood. Not surprisingly, nearly all aspects of cryopreservation protocols- temperature, timing, volumes, containers, cryoprotectants, diluents, stage of embryos, etc. - were established by trial and error in an attempt to balance the large number of opposing factors that affect vitrification and freezing. This review is meant to explain the

principles behind cryopreservation and discuss scientific literature that makes up the current body of knowledge on cryopreservation of bovine embryos.

Temperature

Mazur (1984) demonstrated that cells need to be kept below the glass transition temperature of water (-132 °C) to stop biological activity. This transition actually occurs between -90 °C and -132 °C, but any amount of time above that temperature range can result in recrystallization, or formation of ice which is damaging to cells. As early as 1968, Ackerman showed that human sperm stored at -79 °C on dry ice experienced decreased post-thaw motility; the effect was more pronounced above -75 °C. This sensitivity however, seems to be species-specific, as bull sperm survived upon thawing after 4 years of storage at -79 °C (Leibo, 1999). Nonetheless, it is recommended that cryopreserved cells be stored in liquid nitrogen vapor (<-150 °C) or liquid nitrogen (-196 °C).

Damage caused by Cryopreservation

Thermal shock and ice formation are the main causes of cryoinjury. When cells are cooled too quickly, thermal shock can take place. Ice formation occurs if cells are not allowed to dehydrate properly as freezing occurs. Even though both types of damage can occur at as low as -80 °C, the critical range is between 15 °C and 0 °C. (Cryo Bio System, 2006). Damage of the plasma membrane is the initial step of thermal shock, and is due to mechanical shearing and membrane elements shrinking in diverse manners resulting in conformational changes to the outside of the cell (Cryo Bio System, 2006).

These problems are why optimization of cooling rates is key in successful cryopreservation.

Principles of Freezing

The first report of successfully freezing a mammalian embryo was in 1972 (Whittingham et al.) in the mouse. Not long after, the first live calf was born from embryo transfer of a frozen/thawed bovine embryo (Wilmut and Rowson, 1973). Because conventional slow freezing was developed before vitrification and produced acceptable pregnancy rates after embryo transfer (80-90% of non-frozen controls; Hasler, 2001), freezing became the industry standard for bovine embryo cryopreservation. Usually this technique involves a single cryoprotectant (previously glycerol; more currently ethylene glycol; Voelkel and Hu, 1992b) at concentrations ranging from 1 to 2 M (approximately 10%) often made in modified Dulbecco's PBS + 0.4% BSA (PBS) to limit toxic and osmotic stress. Embryos are allowed to equilibrate for 5 to 20 min at 22 °C. Freezing is accomplished by use of a programmable freezer, sometimes associated with a laptop computer (adding to the expense), and can hold approximately 20 to 100 plastic straws (0.5 mL or 0.25 mL). Cooling occurs at a controlled rate, at around -4 °C per min, or faster, until the temperature reaches -6 °C, at which time straws are seeded (i.e. contact of a metal surface with the plastic straw to initiate crystallization) and cooling is resumed at a rate of -0.3 to -0.6 °C per minute until embryos reach -30 °C and are plunged into liquid nitrogen at -196 °C. Upon thawing, the 0.25 mL straws are held in air for 8 s and then placed in a 37°C water bath for no less than 20 s. If cryopreserved with ethylene glycol, the

embryos are deposited directly into the uterus of a recipient female using an embryo transfer gun (Bracke and Niemann, 1995). Conversely, if glycerol is used as the cryoprotectant, it must be removed from embryos, either in a single step using 1 M sucrose (Leibo et al., 1984) or in a step-wise fashion: e.g. 0.8 M glycerol + 0.3 M sucrose for 6 min, 0.3 M glycerol + 0.3 M sucrose for 6 min, 0.3 M sucrose for 6 min, and PBS for 2 min. After cryoprotectant removal, embryos are loaded into 0.25 mL straws for nonsurgical embryo transfer.

The controlled cooling rate associated with conventional freezing allows intra- and extracellular water exchange to occur without serious osmotic effects (Vajta and Kuwayama, 2006), and formation of extracellular ice is what allows the cells to dehydrate. Dehydration is important because it prevents damage from intracellular ice formation. Although acceptable pregnancy rates (80-90% of non-frozen controls; Hasler, 2001) result from embryos cryopreserved via conventional freezing, cryopreservation presents some challenges: 1) freezing equipment is costly, 2) protocols typically take over an hour, 3) ice crystal formation can damage embryos, and 4) technical difficulties with seeding can occur.

Principles of Vitrification

Fahy (1984) defined vitrification as the solidification of a solution brought about not by crystallization but by extreme elevation in viscosity during cooling. Quite literally, vitrification means to make glass, given the Latin root word *vitri/vitrium* meaning glass. Rall and Fahy (1985) were the first to report

successful vitrification of mammalian embryos, those of mice. Cryopreservation without the formation of damaging ice crystals is the foremost advantage of vitrification over freezing. Other distinct aspects of vitrification include; 1) an elevated level of cryoprotectant and increased viscosity in the vitrification medium, 2) rapid heat exchange, 3) small volume used to vitrify cells, and 4) minimal time and cost associated with this technique. Although hundreds of vitrification studies have been published (Vajta and Nagy, 2006) since the groundwork done by Rall and Fahy, there has been little application of the technique in commercial bovine embryo transfer. In 2005, less than 0.2% of commercially cryopreserved bovine embryos in the United States were vitrified (American Embryo Transfer Association, 2006). In more recent years, that rate has remained relatively constant with less than 0.3% (456/162,812) of embryos in the United States being vitrified in 2009 (American Embryo Transfer Association, 2010). Better understanding of the technology, leading to simplification and efficiency improvements should allow for greater adoption of the technology industry-wide (Brad Stroud, DVM Stroud Veterinary Embryo Service, Inc., personal communication).

Several vitrification protocols exist for bovine embryos (Martinez et al., 1998; Park et al., 1999; Kaidi et al., 1999; Nguygen et al., 2000; Kaidi et al., 2000; Martinez et al., 2002), but a consensus on the key steps is being slowly realized. Arguably one of the most convincing studies conducted on a large scale was done by van Wagtendonk de Leeuw et al. (1997) in which 728 in vivo-produced bovine embryos were cryopreserved in 0.25 ml straws either by

vitrification or conventional slow freezing, with nearly identical resulting pregnancy rates (44.5% and 45.1%, respectively). Seidel and Walker (2006) based the following protocol on the aforementioned study with slight modifications: *Expose embryos to 5 M ethylene glycol in base medium for 3 min. Transfer embryos in ≤1 µl into a 15 µl drop of vitrification solution containing 7 M ethylene glycol + 18% w/v Ficoll 70 + 0.5 M galactose in base medium for 45 s. While in vitrification solution, aspirate a column of diluent (1 M galactose in base medium) followed by air, more diluent, air, vitrification solution containing embryos, air, diluent, and finally an air space at the end of the straw. Plug the straw, and after 45 s, place into goblet in liquid nitrogen. Hold straw in vapor for 1 to 3 min and plunge in liquid nitrogen. Embryos are then warmed by holding in air for 8 s followed by a 37 °C water bath for 15 s. Straws are shaken like a clinical thermometer four times to mix the diluent with vitrification solution. Embryos can be held for 5 to 10 min in air or in 37 °C water until transfer.*

Elevated Cryoprotectant Concentrations

MacFarlane (1987) demonstrated that vitrification occurs most optimally when cryoprotectant concentration is higher than 40% v/v. This elevated level of cryoprotectant, when compared to conventional slow freezing, approaches the limits of osmotic and toxic stress. Therefore, cells can only be exposed to such high concentrations of cryoprotectants for a short time, and must be cooled quickly. One option is to use mixtures of two cryoprotectants, as demonstrated by Liebermann et al. (2003) who combined 20% ethylene glycol and 20% DMSO for cryopreservation of 1,120 human oocytes, resulting in 80.8% survival.

However, studies involving combinations of cryoprotectants for vitrifying bovine embryos have not proven to be more effective than a single cryoprotectant. Van Wagtendonk-De Leeuw et al. (1995) compared in vivo embryos vitrified in either 46% glycerol or a combination of 25%glycerol and 25% propanediol with resulting pregnancy rates of 43% (17/40) and 24% (5/21), respectively; ($P > 0.1$). Martinez et al. (1998) also experienced similar results when he compared pregnancy rates after transfer of embryos vitrified in either 40% ethylene glycol or a combination of 25% glycerol and 25% ethylene glycol with pregnancy rates of 35% (6/17) and 44% (7/16), respectively; ($P > 0.1$). None of the previously mentioned studies had a large enough sample size per treatment to make any definite conclusions about the use of single versus multiple cryoprotectants. Upon warming, cryoprotectants are typically removed in the presence of sucrose (MW = 342.30) or galactose (MW = 180.16).

Significance of Viscosity

One of the most important aspects of whether a solution will either freeze or vitrify is viscosity, which is greatly increased in vitrification solutions based on the simple principle that the closer the solution is to a solid, the more quickly it will turn to glass. This is done with the addition of macromolecules to vitrification solutions, and can include polyethylene glycol (MW = 8,000), Ficoll/Ficoll70 (MW = 400,000/70,000), or polyvinylpyrrolidone (PVP) (MW = 360,000). The increased viscosity allows vitrification to occur with a lowered concentration of cryoprotectant (27% w/v ethylene glycol; Kuleshova et al., 2001). Additionally, macromolecules prevent disruption of the zona pellucida during vitrification, with

embryos vitrified in solutions containing PVP experiencing 4.8% zona damage versus 20.4% for those without PVP ($P < 0.01$) (Titterington et al., 1995).

Rapid Heat Exchange

Unlike conventional freezing methods, vitrification works via the principle of ultra-rapid cooling; cells are cooled at rates of up to 20,000 °C per min (Vanderzwalmen et al., 1997) versus 0.3 to 0.6 °C per min with conventional freezing. Cooling and warming rates can be altered by changing the volume of vitrification solution surrounding cells, thickness of the straw wall or other container, and the insulting layer of liquid nitrogen gas that is formed when liquid nitrogen boils. The vitrification solution containing the cells cools so quickly that no ice crystal formation occurs, but instead the highly viscous medium turns to glass due to an elevated concentration of cryoprotectant and presence of macromolecules. Essentially, there is no time for the molecules to reorganize themselves into a crystalline structure. Upon vitrification, cells are in a state of disequilibrium so less damage due to solution effects occurs than those cryopreserved via slow freezing. Another technique associated with vitrification that allows rapid cooling to occur is the small volume of vitrification solution used to cryopreserve cells. The smaller the volume, the larger the surface to volume ratio, and the more quickly the solution will cool and vitrify; it will warm more quickly as well. However, reducing vitrification solution volumes to less than 1 µl, a technically challenging task, seemed not to improve hatching rates of IVF-produced bovine embryos (58.3% in 1 µl versus 61.3% in 0.5 µl; Rios et al., 2010).

Reduced Expense

An added advantage of vitrification is that it does not require a freezing machine. Therefore, equipment costs are minor in comparison to freezing. Only liquid nitrogen itself, liquid nitrogen vapor or cooled air, or an extremely cold (< 150 °C) surface is needed for vitrification. Furthermore, whereas freezing protocols can take upwards of an hour, vitrification takes only a few min. One caveat, however, is that since embryos are vitrified one after the other, the process can take longer if there are numerous embryos. Also, because current procedures for cryopreservation of in vivo-produced bovine embryos result in acceptable pregnancy rates, vitrification would be most advantageous for embryos that do not cryopreserve well. Cryopreservation is problematic for in vitro-produced embryos, and in vivo-produced embryos from Bos indicus and Jersey cattle (Steel and Hasler, 2004).

Studies Comparing Vitrification and Conventional Freezing

A number of cryopreservation studies compare methods of vitrification that do ***not*** allow for direct transfer with conventional slow freezing. One example is a study by Xu et al. (2006), which evaluated pregnancy rates of recipients receiving embryos either; 1) produced in vitro, fertilized by sexed semen and vitrified via contact with a cold metal surface; 2) fresh, in vitro-produced embryos, fertilized by conventional (unsexed) semen; and 3) in vivo-produced embryos frozen conventionally, resulting in pregnancy rates of 40.9%, 41.9%, and 53.1%, respectively. Numerous other such studies exist, but are beyond the scope of

this review. Furthermore, due to the expense of purchasing and maintaining cattle, the majority of the aforementioned studies rarely include pregnancy and calving rates following embryo transfer, but consider survival rates after in vitro culture as a measure of success. For simplicity and a more direct comparison of what is realistic in the cattle embryo transfer industry, see **Table 2.1,** which summarizes publications regarding pregnancy rates of recipients after transfer of vitrified or frozen IVF or in vivo-produced bovine embryos. Most of the studies cited likely involved embryos highly selected for morphological normality.

Table 2.1. Summary of publications regarding embryos transferred after vitrification in 0.25 mL straws

Reference	Treatment	Cryoprotectant	D ŧ	Pregnancy Rate
Tachikawa et al., 1993¥	Vitrified IVP*	40% glycerol	S	13% (1/8)
	Vitrified IVP	50% ethylene glycol	S	25% (5/20)
	Fresh IVP	N/A	N/A	23% (5/22)
van Wagtendonk-De Leeuw et al., 1995	Vitrified In Vivo	46% glycerol	IS	43% (17/40)
	Vitrified In Vivo	25% glycerol/25% propanediol	IS	24% (5/21)
	Slow Frozen In Vivo	9% glycerol	S	59% (20/34)
Saha et al., 1996	Vitrified In Vivo	40% ethylene glycol	IS	60% (3/5)
Vajta et al., 1997	Asst'd H'g Vitrified IVP	25% ethylene glycol/25% DMSO	IS	35% (7/20)
van Wagtendonk-De Leeuw et al., 1997	Vitrified In Vivo	48% glycerol	IS	44.5% (174/393)
	Slow Frozen In Vivo	10% glycerol	S	45.1% (151/335)
Agca et al., 1998a	Biopsied Vitrified IVP	25% glycerol/25% ethylene glycol	S	44% (7/16)
	Biopsied Slow Frozen IVP	11% glycerol	Sl	23% (3/13)
	Biopsied Fresh IVP	N/A	N/A	50% (7/14)
Agca et al., 1998b§	Vitrified IVP	25% glycerol/25% ethylene glycol	S	38% (12/32)
	Slow Frozen IVP	10 % glycerol	S	26% (9/34)
	Fresh d6 IVP	N/A	N/A	59% (47/80)
	Fresh d7 IVP	N/A	N/A	54% (38/70)
Donnay et al., 1998	Vitrified IVP	25% glycerol/25% ethylene glycol	S	9% (1/11)
	Vitrified IVP	10% glycerol/40% ethylene glycol	S	0% (0/11)
Martinez et al., 1998	Vitrified IVP	40% ethylene glycol	S	35% (6/17)
	Vitrified IVP	25% glycerol/ 25% ethylene	S	44% (7/16)
	Fresh IVP	glycol	N/A	36% (5/14)
		N/A		
Pugh et al., 2000	Vitrified IVP	20 % ethylene glycol/20% DMSO/10% 1,3-buteanediol	IS	22% (17/76)
	Fresh IVP	N/A	N/A	22% (5/22)
Al-Katanani et al., 2002 ₸	Vitrified IVP	25% glycerol/25% ethylene glycol	S	6.5% (3/54)
	Fresh IVP	N/A	N/A	19% (6/33)
Martinez et al., 2002	Vitrified IVP 0.1M sucrose	25% glycerol/25% ethylene glycol	S	50% (20/40)
	Vitrified IVP 0.3M sucrose	25% glycerol/25% ethylene glycol	S	40% (16/40)
	Fresh In Vivo	N/A	N/A	65% (26/40)
	Fresh IVP	N/A	N/A	51% (18/35)
Nedambale et al., 2004	Vitrified IVP	48% glycerol	S	30% (3/10)
Wurth et al., 1994	Vitrified IVP	48% glycerol	IS	24% (20/85)
	Slow Frozen IVP	9% glycerol	S	14% (5/35)
	Fresh IVP	N/A	N/A	42% (52/121)
Kruse, unpublished (2011)	Vitrified IVP	36% ethylene glycol	IS	14% (11/80)
	Slow Frozen IVP	10% glycerol	S	16% (13/80)
	Vitrified In Vivo	36% ethylene glycol	IS	46% (21/46)
	Slow Frozen In Vivo	10% glycerol	S	39% (17/44)

ŧ D= Dilution; S= Sequential; IS = In Straw; *IVP = *in vitro*-produced embryos

¥ Two embryos transferred/recipient; pregnancy rate in terms of number of live calves born/embryo transferred

§Two embryos transferred/recipient; one ipsilateral and one contralateral to the corpus luteum; pregnancy rate in terms of pregnancies/embryo transferred

₸ Timed transfer in lactating dairy cow recipients experiencing heat stress

Adapted from Seidel and Walker, 2006

Cryoprotectants

Widespread use of DMSO (MW = 78.13), glycerol (MW = 92.10), and propylene glycol (MW = 76.10) as cryoprotectants was common until about 1990 (Massip, 2001) when use of ethylene glycol became dominant, although the latter was established as a useful cryoprotectant for freezing mouse and rat embryos many years earlier (Miyamota and Ishibashi, 1977). The low molecular weight (62.07) of ethylene glycol contributes to its highly membrane permeable characteristics, and therefore no stepwise dilution is needed post thawing (Bracke and Niemann, 1995). Ethylene glycol was proven as an effective cryoprotectant at 1.5 M for bovine embryos (Voelkel and Hu, 1992b), and pregnancy rates for in vivo embryos cryopreserved this way were between 50 and 70% (Niemann, 1995). Sommerfeld and Niemann (1999) proved that ethylene glycol was lowly toxic for use in both freezing and vitrification protocols with concentrations ranging from 1.8 to 8.9 M. Nibart and Humblot (1997) studied pregnancy rates of 4,846 bovine embryos frozen in glycerol/sucrose (d 60 pregnancy rate = 48.5%) and 1,239 bovine embryos frozen in 1.5 M ethylene glycol (d 60 pregnancy rate = 50.5%). Although pregnancy rates did not differ ($P > 0.1$), because ethylene glycol moves in and out of cells more quickly, transfer of embryos is easier and more efficient, (allowing for direct transfer) and is currently the industry standard.

Not only is the type of cryoprotectant important for cryopreservation, but also the way in which it is added to embryos is critical. Kuwayama et al. (1992)

performed an experiment adding glycerol/1, 2-propanediol at 45% v/v in 1, 2, 4, 8, or 16 steps for a total of 18 min of equilibration resulting in survival rates of embryos at 24 h of 56, 89, 100, 100, and 100%, respectively. He proposed that step-wise addition of cryoprotectant, although inconvenient, minimized osmotic stress and damage to cells. Walker et al. (2006) tried to simplify the addition of ethylene glycol to a single step and demonstrated survival rates that were not different ($P > 0.1$) from addition in two steps; 85% and 98%, respectively. However, re-expansion rates were lower for one-step addition of cryoprotectant to morulae, blastocysts, and expanded blastocysts (51%, 58%, and 51%, respectively versus two-step addition 55%, 75%, and 89%, respectively). They believed toxicity or osmotic stress due to sudden movement of water and solutes caused the poorer performance of those embryos which had cryoprotectant added in only one step.

Another important element of cryoprotectants is the base medium in which they are made. Scientists certainly value the ability to define components in freezing and vitrification medium not only to avoid contamination, but also to be able to consistently reproduce these solutions without batch differences in undefined additives. This drove replacement of bovine serum albumin with chemically defined macromolecules for freezing. Seidel et al. (1990) showed no difference in survival rates when replacing BSA with polyvinyl alcohol or sodium hyaluronate.

Vitrification Containers, volumes, and heat transfer

The first embryos were frozen in glass ampules (Whittingham et al., 1972). In 1979, the 0.25 mL plastic insemination straw was introduced as a freezing container (Massip et al.). Plastic straws were important because they were easier and safer to store than traditional glass ampules. This also increased cooling rates and allowed diluent (sucrose in this case) to be stored in the straw separated by an air bubble which, when mixed, allowed cryoprotectant to be removed from embryos prior to direct nonsurgical transfer (Leibo, 1984). The change from glycerol to ethylene glycol as a cryoprotectant further facilitated the convenience of direct transfer (Voelkel and Hu, 1992b). The smaller molecule can move in and out of cells more rapidly, allowing dilution to occur in the uterus of recipients. Another plus was that a smaller radius speeds heat transfer and distributes it more evenly throughout cells. Mortimer (2004) examined this phenomenon and proved slow heat transfer to be a major weakness of cryovials, resulting in reduced survival rates of cells (sperm in this case) frozen in large diameter packaging. Measurement of cooling curves in 0.5 and 0.25 mL straws validated that cells packaged in either container undergo similar rates of heat exchange (Cryo Bio System, 2006). Non-sealed packaging devices arose from the necessity of rapid cooling and warming rates and shortened periods of exposure to high concentrations of cryoprotectant, both keys to successful vitrification. Procedures for use of these devices are based on direct exposure of small volumes of vitrification solution containing cells to liquid nitrogen.

The first attempt at non-sealed packaging was use of electron microscope grids containing in vitro-matured bovine oocytes in < 1 µL of vitrification solution. These oocytes resulted in 30% cleavage after fertilization upon warming; half of those developed into blastocysts, and rates were not different from control oocytes exposed to vitrification solution, but not cryopreserved (Martino et al., 1996). Vajta et al. (1998) described an approach termed the Open Pulled Straw (OPS) method, simply heating and pulling a 0.25 mL straw until the inner diameter and wall thickness are reduced to half, resulting in cooling and warming rates which exceed 20,000 °C per min, while embryos are exposed to high concentrations of cryoprotectants for less than 30 s. Both oocytes and blastocysts vitrified via OPS resulted in pregnancies following embryo transfer. One year later, the cryoloop device (comparable to a bubble wand over which a thin film of vitrification solution containing embryos can be stretched) was used to vitrify mouse and human blastocysts successfully without affecting reexpansion or hatching in culture when compared to non-vitrified controls (Lane et al., 1999). Vanderzwalmen et al. (2000) devised the hemi-straw carrier system, essentially a beveled open ended 0.25 mL straw, which allowed successful vitrification of oocytes and blastocysts, resulting in pregnancy following embryo transfer. Arguably the most important contribution to containerless methods was made by Kuwayama et al. (2005), who developed the Cryotop method, in which cells are contained in < 0.1 µL of vitrification solution on the surface of a fine polypropylene strip attached to a plastic handle. Oocyte survival rate was superior with the Cryotop method when compared to vitrification in 0.25 mL

straws or the OPS system. Ninety-one percent (58/64) of bovine oocytes appeared normal after vitrification and warming.

Equally important to cooling rates are rapid warming rates, but such small volumes and limited mass of containers present another problem: increased risk of damage to cells. A 0.25 mL straw will warm to -80 °C (Because glass transition occurs between -132 °C and -90 °C, once temperatures reach -80 °C, damage has already occurred) within 15 s at room temperature (Tyler et al., 1996). Not only do containerless methods warm even more quickly, but they can present challenges as they require more skill to handle prior to vitrification and when dealing with embryo recovery. A microscope is also required for placing embryos into a straw for embryo transfer.

Another concern associated with non-sealed packaging devices is direct exposure of vitrification solutions containing cells to liquid nitrogen. Such exposure could result in cross-contamination from liquid nitrogen. This issue arose in 1995 when an incident occurred involving six cases of acute Hepatitis B virus infection among multiple-transfused patients going through cytotoxic treatment (Tedder et al., 1995). This has been the only reported case of cross-infection due to liquid nitrogen storage to date. However, it prompted further investigation into possible pathogens in liquid nitrogen. Bielanski et al. reported that liquid nitrogen contaminated by pathogenic viruses did not contaminate cells stored properly in containers (2000) nor did micro-organisms in properly sealed containers leak out to contaminate clean cells in the same tank (2003). Further proof that this may be a minimal risk, yet have very serious consequences if it

were to occur, is evident in a 1985 case. Several women became infected with HIV after insemination by an unknown HIV-positive donor, but no infections resulted from inseminations of semen stored in the same cryotank as the infected donor (Stewart et al., 1985). Vapor storage has been proposed as a solution to cross-contamination; even so, not only is a vapor storage system more technically challenging to maintain, but microbial pathogens have been isolated from liquid nitrogen vapor as well (Fountain et al., 1997).

In Vivo- versus In Vitro-Produced Embryos

The first offspring resulting from mammalian IVF were reported in 1959 (Chang, 1955; 1968) in the rabbit. It was over 20 years later (1981) when the first live calf as a result of embryo transfer (surgically into the oviduct of the recipient) of an IVF embryo (four-cell) was born (Brackett et al., 1982). Advances in IVF embryo production and direct nonsurgical transfer have resulted in the births of hundreds of thousands of calves since then.

In 2009, 307,212 bovine in vitro-produced embryos were transferred, and only 7% of those (22,766) were cryopreserved compared with the 55% (297,677/539,683) frozen/thawed in vivo-produced embryos transferred (Stroud, 2010). This considerable difference is not surprising taking into account that successful superovulation, collection, and cryopreservation of in vivo-produced bovine embryos has been common for a number of years, yielding pregnancy rates 80-90% of fresh transferred embryos (Hasler, 2001). Nonetheless, the number of transfers of in vitro-produced embryos has been steadily increasing

since 2000 (Stroud, 2010). Large scale embryo transfer studies have also been conducted yielding acceptable pregnancy rates for fresh IVF bovine embryos (53.8% (1,220/2,268); Hasler et al., 1995). In vitro production of embryos can result not only from mass production from abattoir-derived ovaries, but also those oocytes harvested from ill or infertile genetically valuable females via transvaginal oocyte aspiration or ovariectomy. Furthermore, IVF-produced embryos can serve as an alternative to their much more expensive in vivo-produced counterparts, especially desirable in situations where embryo transfer results in higher pregnancy rates than artificial insemination. This is true in the case of heat stressed or high producing dairy cattle for example (Rasmussen, 2011).

Nonetheless, efficiency of cryopreservation is greatly determined by the origin of the embryos i.e., in vivo- versus in vitro-produced. Even though superior survival upon thawing as well as higher pregnancy rates of in vivo versus in vitro-produced embryos are generally accepted as fact, a recent study by Lonergan et al. (2003) offers more insight. Bovine zygotes produced in vitro were cultured either in vitro in SOF, in vivo in the ewe oviduct, or in a combination of the two to assess the ability of blastocysts to withstand cryopreservation by vitrification. Blastocysts were warmed, and survival was evaluated after culture for 72 h. Those produced after culture in vivo for 6 d had the highest rates of survival (> 95%) versus those cultured in vitro for 6 d (< 20%) followed by the embryos that spent the last 4 d of culture in vivo (73.7%). Those that spent the first 4 d in vivo

and the last 2 d in vitro had 40.6% survival; whereas those that spent only 2 d in vivo followed by 4 d of in vitro culture had the lowest survival rate (6.7%).

The reasons for the disparity in survival rates of in vitro and in vivo embryos are complex. The two types of embryos differ in a number of ways including morphology, cell counts, inner cell mass to trophoblast cell ratio, density, metabolism, lipid content, osmotic behavior, and properties of the zona pellucida (Pollard and Leibo, 1993).

Inner Cell Mass: Trophoblast Cells

Iwasaki et al. (1990) evaluated the percentage of inner cell mass to trophoblast cells in bovine embryos. Embryos cultured in vitro had fewer total cells as well as a significantly lower proportion of inner cell mass in both early (15.8%) and expanded (14.9%) blastocysts when compared to those cultured in vivo (23.4% and 20.8%, respectively). Du et al. (1996) performed a study which was in agreement with inner cell mass differences, but could not reproduce the discrepancy in total cell numbers.

Lipid Content

Leibo et al. (1993) demonstrated that in vitro-produced embryos exhibited an increased sensitivity to chilling and freezing, likely due to the higher lipid to protein ratio than in vivo- produced embryos. Use of centrifugation to remove lipids decreased sensitivity of in vitro-produced embryos to chilling (Leibo, et al., 1995). In vitro survival of frozen/thawed embryos that underwent this delipidation process was improved (delipidated (n = 73; 56.2% survival at 48h) versus control

(n = 67; 39.8% survival at 48h; P < 0.02), but embryo transfer resulted in very poor pregnancy rates (delipidated = 10.5% versus control = 22.4%; P > 0.05) (Diez et al., 1996). Lipid differences may also be the reason for poorer pregnancy rates after cryopreservation of embryos derived from Jersey versus Holstein donors (Steel and Hasler, 2004).

Media and Serum

A further complication in assessing the discrepancies between embryos is that different media and serum (varying in concentration, species derivation, components, and hormones added) can yield in vitro-produced embryos with different properties. Effects of differences in culture media on resulting embryos are quite complex and beyond the scope of this review. One example to illustrate this is a study by Van Soom et al. (1996) that demonstrated that embryos cultured in Ménézo's B2 medium cleaved faster (P < 0.01) and had earlier blastocysts formation (P < 0.06) than those cultured in TCM 199. Also, those embryos cultured in Ménézo's B2 medium had a greater total cell count (24, 65, and 109 cells at d 5, d 6, and d 7, respectively, versus 18, 41, and 71 cells at the same stages when embryos were cultured in TCM 199; P < 0.001). An even larger effect on embryo development is due to culture in media supplemented with serum. When SOF medium was supplemented at d 5 with bovine serum albumin (BSA), fetal calf serum (FCS), or charcoal-treated FCS (CT-FCS), blastocyst development varied (21.6%, 40.1%, and 39.4% blastocysts from cleaved embryos for BSA, FCS, and CT-FCS, respectively; P < 0.01) while cell number remained similar. Pregnancy rates at d 50 following embryo transfer

tended to be lower for embryos cultured in FCS (FCS = 37.7% versus BSA = 53.3% and CT-FCS = 57.6%; P = 0.1) (Thompson et al., 1998).

Stage of Embryo Development

A recent review by Liebermann (2009) states that vitrification and transfer of blastocyst-stage human embryos is the preferred method among human fertility clinicians due to increased pregnancy rates, improved selection of potentially viable embryos, and flexibility of laboratory staff to cryopreserve individual blastocysts based on optimal expansion and development. In a five year study at the Fertility Centers of Illinois, embryo transfer of vitrified human blastocysts resulted in a 96.3% (2730/2835) survival rate, 29.4% implantation rate, and a clinical pregnancy rate per vitrified (multiple) embryo transfer of 42.8% (599/1398) (Liebermann, 2009).

When discussing bovine embryos though, the consensus is that for in vitro-produced embryos, d 7 expanded blastocysts are ideal for cryopreservation (Hasler et al., 1997; Sommerfeld and Niemann, 1999). Examples include Han et al. (1994) who demonstrated a difference in survival of frozen-thawed embryos between blastocyst developmental stages (early vs. mid, $P<0.05$; mid vs. expanded, $P<0.01$; early vs. expanded, $P<0.001$); also, post-thaw survival of blastocysts frozen at d 7 of culture was higher than those reaching the blastocyst stage on d 8 (62% and 45%, respectively). Vajta et al. (1996) also showed that more advanced stage blastocysts are optimal for post-vitrification development. Hatching rates upon warming increased as stage of the embryo advanced with

morulae, early blastocysts, blastocysts, and expanded blastocysts resulting in hatching rates of 10%, 34%, 47%, and 63%, respectively. Studies by Pugh et al. (2000) demonstrated that in vitro-produced bovine blastocysts are more robust than morulae as they had both greater survival at 24 h (75% and 24%; $P < 0.001$) and hatching at 48 h (59% and 15%; $P < 0.001$) as well as d 90 pregnancy rates (53.8% (14/26) and 17.4% (4/23); $P < 0.02$).

In contrast, in vivo produced embryos tend to result in more pregnancies if transferred at earlier stages. Hasler et al. (1987) showed that embryo transfer of in vivo-produced bovine embryos at early blastocyst and mid-blastocyst stages resulted in higher pregnancy rates than morulae, expanded blastocysts, or hatched blastocysts. Data in **Table 2.2** include embryo production (IVF versus in vivo), stage of development, and cryopreservation status (fresh or frozen), and support the aforementioned interactions (Hasler, 1998). Pregnancy rates ranged from 67 to 20% with transfer of d 7 fresh in vivo embryos resulting in the highest pregnancy rate followed by d 7 frozen in vivo, d 7 fresh IVF, d 8 fresh IVF, d 8 frozen IVF, d 9 fresh IVF, and d 8 frozen IVF.

Table 2.2. Pregnancy rates following transfer of fresh and frozen bovine in vivo- and IVF-derived embryos into Holstein heifers at one location

			Percent Pregnant			
Embryo	**Embryo age, d**	**No. of transfers**	**Embryo Grade 1**	**Embryo Grade 2**	**Embryo Grade 3**	**All Grades**
IVF-fresh	7	4,606	56[a]	41[a]	-	54[a]
IVF-fresh	8	462	48[b]	30[b]	-	43[b]
IVF-fresh	9	22	41	-	-	41
IVF-frozen	7	67	42[b]	-	-	42[b]
IVF-frozen	8	30	20[c]	-	-	20[c]
In Vivo-fresh	7	599	76[d]	67[c]	56	67[d]
In Vivo-frozen	7	517	64[e]	-	-	64[d]

[a,b,c,d,e] Values within a column with different superscripts differ (P < .05), **Adapted from Hasler, 1998**

Species Considerations

A very valid argument for cryopreservation of gametes and embryos is for conservation of animal genetics, with particular concern for loss of genetic diversity in the domestic livestock population. Nearly 20% of the world's breeds of cattle, pigs, goats, horses, and poultry currently are at risk of extinction according to the FAO (2007). Reasons for conservation of livestock species include advantages associated with heterosis, surmounting selection plateaus, biosecurity to maintain a safe food supply, and insurance against changes in climate, availability of feedstuffs, disease, and other catastrophes (Prentice and Anzar, 2011). Although cattle have been the central focus of this review, offspring have resulted following embryo transfer of frozen/thawed embryos from sheep, goats, horses, and pigs (Massip, 2001). A summary of embryos recently transferred by species can be found in **Table 2.3**.

Table 2.3. Numbers of Embryos Transferred by species in 2009 (Ranked by Embryos Flushed)

Species	Transferrable Embryos	Embryos Transferred	Fresh	Frozen
Cattle	1,083,000	843,862	528,331	315,531
Sheep	32,768	1,734	1,326	408
Horses	24,515	24,470	24,455	15
Goats	2,478	352	206	146
Swine	1,498	780	780	0
Cervids	953	941	941	0

Adapted from Stroud, 2010

Ovine

Sheep embryos respond similarly to bovine embryos, and to DMSO as a cryoprotectant (Willadsen, 1977). Since the earliest published studies, ovine embryos have been vitrified successfully (Gajda et al., 1989). Work by Traidi et al. (1999) shows that in vitro-produced goat embryos appear to withstand vitrification better than in vitro-produced sheep embryos, with in vitro survival being 60% (106/177) for goats versus 41% (51/124) for sheep. Pregnancy rates were 45% (9/20) in goats and 15% (5/34) in sheep. Cryopreservation methods and subsequent pregnancy rates have improved in recent years as evident by research of Green et al. (2009), who produced pregnancy rates of 50.0%, 38.6%, and 55.8% for fresh, frozen, and vitrified ovine embryos, respectively ($P > 0.1$).

Porcine

Cryopreservation of porcine embryos has been the most challenging of the livestock species. This is partially because scientists believed for a number of years that embryos could not be exposed to temperatures less than 15 °C without experiencing significant cryoinjury (Polge, 1977). Furthermore, porcine embryos have a much higher lipid content than other species, making cryopreservation more difficult. Nonetheless, porcine embryos at certain stages with various treatments have been successfully frozen and transferred resulting in piglets (Hayashi et al., 1989) and vitrified by use of OPS resulting in pregnancy (Berthelot et al., 2000) and excellent in vitro survival (Holm et al., 1999).

Equine

Equine embryos present a unique challenge due to their size and capsule formation, which may impair movement of cryoprotectant into the embryo. In fact, size and developmental stage of the embryo are more important to embryo survival after freezing than type of cryoprotectant (Squires et al., 1999). Even so, freezing protocols are similar to those for bovine embryos, and acceptable pregnancy rates (53%; Slade et al., 1985; 50%; Lagneaux et al., 1998) can be achieved for smaller embryos. Pregnancies also have been reported for vitrified equine embryos (Hochi et al., 1994), and a direct transfer protocol has been developed with excellent success rates using early blastocysts (Eldridge-Panuska et al. 2005).

Chapter 3

Experiment 1. Lowering sodium and calcium concentrations in vitrification media and cooling straws in liquid nitrogen cooled air versus contact with an aluminum block submerged in liquid nitrogen to improve survival rates of in vitro-produced vitrified bovine blastocysts

Introduction

In vitro-produced bovine embryos can be created from abattoir-derived ovaries at low cost, but practical use requires effective cryopreservation that allows embryos to be transferred directly to the uterus of recipients on farm. Such embryos are in demand for both beef and dairy cattle, as embryo transfer can result in pregnancy after failure of artificial insemination to result in pregnancy of heat stressed animals or high producing dairy cattle (Rasmussen, 2011). Unfortunately, standard slow cooling methods do not work well for in vitro-produced embryos (Agca et al., 1998a; Agca et al., 1998b; Wurth et al., 1994). Vitrification offers promising post-warming survival rates (Vajta and Nagy, 2006).

Vitrification is an alternative method to freezing for cryopreservation of embryos. Rall and Fahy (1985) were the first to successfully vitrify mammalian embryos in the mouse. With vitrification, cryopreservation occurs much more rapidly than with conventional slow freezing, eliminating ice crystals and the

intracellular damage they may cause. Additionally, vitrification is relatively simple, and eliminates the need for costly freezing equipment. However, embryos must be exposed to high concentrations of cryoprotectant for solutions to vitrify (Massip, 2001). Exposing embryos to only a small volume of medium using containerless methods facilitates vitrification, but such procedures are impractical for on farm use. Plastic straws are the most convenient containers for cryopreservation because they are used for direct transfer.

Our laboratory previously developed practical procedures by optimizing cryoprotectant concentrations, placing standard semen straws in liquid nitrogen cooled air for initial cooling, and optimizing timing of the various steps for vitrifying both equine (Eldridge-Panuska et al., 2005) and bovine (Campos et al., 2006) embryos, with the advantage that straws can be used to enable direct transfer. Excellent pregnancy rates have been achieved for early stage equine embryos and in vivo-produced bovine embryos, but not for the more delicate in vitro-produced bovine embryos. Effective and practical vitrification methods are needed for these embryos as well. In 2009, 307,212 bovine in vitro-produced embryos were transferred worldwide, but only 7% of those (22,766) were frozen due to poor survival of such embryos after thawing. In contrast, the majority of in vivo-produced embryos transferred were frozen/thawed (55%; 297,677/539,683) (Stroud, 2010). This considerable difference is not surprising, taking into account that successful superovulation, collection, and cryopreservation of in vivo-produced bovine embryos has been common for a number of years, yielding pregnancy rates 80-90% of those of fresh transferred embryos (Hasler, 2001).

Nonetheless, the number of transfers of in vitro-produced embryos has been steadily increasing since 2000 (Stroud, 2010). Large scale embryo transfer studies have also been conducted yielding acceptable pregnancy rates for fresh IVF-produced bovine embryos (53.8%; 1,220/2,268); Hasler et al., 1995). Therefore, the objective of this experiment was to improve vitrification procedures for in vitro-produced bovine blastocysts such that post warming survival is maximized, making this procedure practical by enabling direct transfer.

Larman et al. (2006) showed that ethylene glycol caused a transient increase in intracellular calcium concentration in mouse oocytes, which was reduced by removal of extracellular calcium in vitrification medium. Furthermore, vitrification of embryos in calcium-free media reduced zona hardening and increased cleavage upon warming. Additionally, calcium-induced apoptosis could theoretically be alleviated by removing or reducing calcium in vitrification media.

Sodium concentration is another potential problem leading to solution effects and toxicity. Stachecki et al. (1998) demonstrated that survival, fertilization, and development were inversely related to the concentration of sodium in freezing medium, and that choline substitution resulted in the highest survival rates upon warming vitrified mouse oocytes. Oocytes are more difficult to cryopreserve than embryos and we believe choline substitution could be beneficial in embryo cryopreservation as well. Therefore, we hypothesized that more rapid cooling and lowering sodium and calcium concentrations in vitrification media would improve post warming embryo survival rates.

Materials and Methods

In Vitro Embryo Production

Expanded bovine blastocysts were produced by standard in vitro procedures developed by our laboratory (de la Torre-Sanchez et al., 2006). Briefly, cumulus-oocyte complexes were obtained by aspirating follicles from abattoir-derived ovaries of feedlot heifers and matured for 23 h in vitro in chemically defined media containing follicle-stimulating and luteinizing hormones, 17-β estradiol, cystemine, and epidermal growth factor. After maturation, oocytes were fertilized by frozen/thawed semen from one of three bulls (one per replicate). Semen was centrifuged through a 45:90% Percoll gradient (2 mL each) in conical 15-mL test tubes for 20 min at 400 x gravity to separate primarily normal, motile sperm. Concentration was adjusted to 5 x 10^5 sperm/mL and added to medium in wells containing oocytes for fertilization. After 18 h of coincubation of gametes, cumulus-oocyte complexes were vortexed to remove cumulus cells and dead sperm. Presumptive zygotes were cultured in chemically defined medium 1 (CDM-1) for 56 h at which time they were evaluated for cleavage. Those reaching at least the 8+ cell stage were then cultured in CDM-2 for 4 d at which time blastocysts were identified. Only d 7 blastocysts, expanded blastocysts, or hatching blastocysts of excellent or good quality were vitrified.

Vitrification

Standard vitrification procedures developed by our laboratory (Campos et al., 2006) were used with slight modifications. This experiment was designed as 4 treatments, the combinations of 2 base media and 2 vitrification methods. Base medium for all vitrification solutions contained 0.2% PVA and 2.5 µg/ml gentamycin sulfate and was either commercially available Syngro Holding Medium (Bioniche Life Sciences, Belleville, Ont) which contains normal physiological concentrations of sodium (120 mM) and calcium (2 mM) (CON) or a product made specifically for this project (Bioniche Life Sciences, Belleville, Ont) which had lowered concentrations of sodium and calcium (60 mM NaCl + 60 mM choline chloride and 0.5 mM calcium) (LOW). Blastocysts were exposed to 5 M ethylene glycol made in CON or LOW base medium (V1) for 3 min at 22 ± 2 °C and moved in less than 1 µl of V1 into 20 µl of 6.5 M ethylene glycol + 0.5 M galactose + 18% Ficoll made in CON or LOW base medium (V2) at 22 ± 2 °C and immediately loaded into 0.25 mL plastic straws. Straws were loaded with a column of 120 µl 1 M galactose followed by an air bubble, V2 containing embryos followed by an air bubble, and 60 µl 1 M galactose followed by sealing with a plastic plug as shown below:

Figure 3.1. Diagram of straw with 1 M galactose (blue) followed by an air bubble (clear), V2 containing embryos (purple), an air bubble, 1 M galactose, and sealed with plastic plug (black).

After 35 to 40 s in V2, embryos were vitrified by either; 1) standard cooling in a goblet surrounded by liquid nitrogen, and thus containing liquid nitrogen cooled air (AIR) for 1 min or 2) cooling for 2 min via contact of straw walls with columns drilled into an aluminum block that was immersed in liquid nitrogen (BLK) and then directly plunging straws into liquid nitrogen, thus resulting in 4 treatment groups (AIR x CON, 4 replicates, n = 61; AIR x LOW, 5 replicates, n = 58; BLK x CON, 4 replicates, n = 73; BLK x LOW, 6 replicates, n = 54). Semen from 1 of 3 bulls was used in a given replicate. Embryos were then stored in liquid nitrogen at -196 °C for at least 24 h until warmed.

Warming and Culture

Embryos were warmed by holding straws in air at 22 ± 2 °C for 8 s and then placing them in a water bath at 37 °C for 20 s. Straws were then shaken (like a clinical thermometer) to mix embryos with the 1 M galactose diluent in the straw, where they remained for 2 min. Straw contents were then expelled into CON or LOW base medium. Embryos were recovered, rinsed through base medium for 2 min, and moved to CDM-2 to be cultured for 24 h before evaluation for survival. Embryos were evaluated morphologically by reformation of the blastocoele, expansion, and hatching and scored for quality (1 = excellent; 2 = good; 3 = fair; 4 = poor; 5 = degenerate). Embryos of excellent, good, and fair quality were considered to have survived; poor quality and degenerate embryos were not.

Statistical Analysis

For each replicate, the percent survival was calculated for each treatment, and then percent values were subjected to a one-way ANOVA. Each treatment was replicated 4 to 6 times. There were no interactions between bull or replicate and treatment and therefore these variables were not included in the model.

Results and Discussion

Post warming survival did not differ ($P > 0.10$) between treatments (AIR x CON = 42.0%; AIR x LOW = 26.8%; BLK x CON = 21.8%; BLK x LOW = 24.5%). There were no treatment by replicate or bull by replicate interactions.

Survival rates were lower than expected. This likely was due to a number of factors. Embryos were derived from ovaries of feedlot heifers, most of which likely had been fed melengesterol acetate (MGA) prior to slaughter to enhance growth rates and prevent weight loss associated with estrus in the feedlot. Exposure to progestin would prevent heifers from having estrous cycles, and oocytes isolated from such ovaries are subfertile compared to those from culled cows (Barceló-Fimbres et al, 2011). In fact, even heifers fed MGA for synchronization of estrus for more than 9 d are subfertile on the first estrus after withdrawal, which is why prostaglandin F2α is given to induce a second, more fertile estrus before insemination.

Furthermore, there was contamination in our water filtration system at the time this experiment was conducted. All maturation, fertilization, and culture media were made with this suboptimal water, discovered in retrospect.

Blastocyst rates (number of blastocysts produced per oocyte matured) were much lower than normal at this time as well. Upon correction of this contamination problem, blastocyst rates exceeded 30%.

Additionally, less stringent selection parameters were used when choosing blastocysts to be vitrified. Blastocysts, expanded blastocysts, and hatching blastocysts of excellent and good quality were cryopreserved. Subsequent experimentation in our laboratory as well as that of many previously published studies (Han et al., 1994; Vajta et al., 1996; Hasler et al., 1997; Sommerfeld and Niemann, 1999) indicates that the d 7 expanded bovine blastocyst is the most ideal for cryopreservation when embryos are produced in vitro. Later experiments were limited to the use of only excellent quality expanded blastocysts.

Despite lack of statistical significance, we recommend use of LOW base media, as the reduced levels of sodium and calcium should have less chance of both sodium and calcium toxicity, and could deter calcium-induced apoptosis. The BLK vitrification method is both easier to use and more consistent than placing straws in liquid nitrogen-cooled air as straws can simply be placed in the holes in the block and will be cooled in the same manner each time. It also avoids the complications of straws cracking when liquid nitrogen contacts the straw directly at room temperature which occurs occasionally with vapor cooling if goblets are cracked.

Chapter 4

Experiment 2. Evaluation of in vitro post-warming survival after vitrification of in vitro-produced bovine embryos in 0.25 ml straws for direct transfer

Introduction

Multiple protocols for vitrification of bovine embryos exist (Martinez et al., 1998; Park et al., 1999; Kaidi et al., 1999; Nguygen et al., 2000; Kaidi et al., 2000; Martinez et al., 2002; Seidel and Walker, 2006), but procedures that work well in the laboratory are not practical for on farm use. One example is the Cryotop method (Kuwayama et al., 2005) in which embryos are placed in < 0.1 µL of vitrification solution on the surface of a fine polypropylene strip attached to a plastic handle. Although survival is excellent, a microscope and skilled technician are required to transfer embryos to a straw post warming before they can be transferred into a recipient, which is awkward and impractical for on farm application.

To facilitate direct transfer, cryopreservation methods have been developed using the 0.25 mL plastic straw as the freezing container. Several investigators have demonstrated that pregnancies can result from direct transfer of embryos vitrified in 0.25 mL straws (van Wagtendonk-De Leeuw et al., 1995;

Saha et al., 1996; Vajta et al., 1997; van Wagtendonk-De Leeuw et al., 1997; Pugh et al., 2000; Wurth et al., 1994). However, there has been little application of this technology in commercial embryo transfer. In fact, in 2005, less than 0.2% of commercially cryopreserved bovine embryos were vitrified (American Embryo Transfer Association, 2006). That percentage has remained relatively constant in more recent years with only 0.3% (456/162,812) of embryos in the United States being vitrified in 2009 (American Embryo Transfer Association, 2010).

Even though vitrification is not being adopted, in vitro-production of bovine embryos is becoming more popular and has been increasing globally since 2000 (Stroud, 2010). This is attributed to the capacity of an in vitro system to produce a large number of embryos relatively inexpensively from abattoir-derived ovaries plus the ability to obtain large numbers of oocytes via transvaginal aspiration from ovaries of Bos indicus donors. World-wide, 307,212 bovine in vitro-produced embryos were transferred in 2009; only 7% of those were cryopreserved (Stroud, 2010). The reasons that in vivo-produced embryos are more robust than in vitro-produced embryos are many. Put simply, available culture conditions do not mimic the oviduct and uterus closely enough to produce an embryo that survives as well as in vivo-produced counterparts.

A practical and simple cryopreservation method for these more fragile embryos is needed is to further facilitate the demand for pregnancies from in vitro-produced embryos. Therefore, our objective was to compare post warming survival rates of cryopreserved in vitro-produced bovine blastocysts packaged in

0.25 mL straws using a simple cooling method: contact of straw walls with holes in an aluminum block submerged in liquid nitrogen versus conventional freezing. We hypothesized that more rapid cooling and elimination of ice crystals associated with vitrification would enable greater survival rates upon warming and culture.

Materials and Methods

In Vitro Embryo Production

Expanded bovine blastocysts were produced by standard in vitro procedures developed by our laboratory (de la Torre-Sanchez et al., 2006). Briefly, cumulus-oocyte complexes were obtained by aspirating follicles from abattoir-derived ovaries of cull cows and matured for 23 h in vitro in chemically defined media containing follicle-stimulating and luteinizing hormones, 17-β estradiol, cystemine, and epidermal growth factor. After maturation, oocytes were fertilized by frozen/thawed semen from one of three bulls (one per replicate). Semen was centrifuged through a 45:90% Percoll gradient (2 mL each) in conical 15-mL test tubes for 20 min at 400 x gravity to separate primarily normal, motile sperm. Concentration was adjusted to 5 x 10^5 sperm/mL and added to medium in wells containing oocytes for fertilization. After 18 h of coincubation of gametes, cumulus-oocyte complexes were vortexed to remove cumulus cells and dead sperm. Presumptive zygotes were cultured in chemically defined medium 1 (CDM-1) for 56 h at which time they were evaluated for

cleavage. Those reaching at least the 8+ cell stage were then cultured in CDM-2 for 4 d at which time blastocysts were identified. Only d 7 expanded blastocysts of excellent quality were cryopreserved. Although not a method for direct transfer, Cryotops were chosen as a positive control (CON), as they are becoming the industry standard for vitrification of human oocytes and embryos. Embryos were cryopreserved by vitrification with either a Cryotop (CON; n = 118) or an aluminum block (BLK; n = 128), or by slow freezing (SLF; n = 131). Seven replicates were conducted.

Vitrification

Standard vitrification procedures developed by our laboratory (Campos et al., 2006) were used with slight modification. The base medium for all vitrification solutions was a specially formulated version of Syngro Holding Medium (Bioniche Life Sciences, Belleville, Ont), which had lowered concentrations of sodium and calcium (60 mM NaCl + 60 mM choline chloride and 0.5 mM calcium). Blastocysts were exposed to 5 M ethylene glycol made in base medium (V1) for 3 min at 22 ± 2 °C and moved in less than 1 µl of V1 into 20 µl of V2 (6.5 M ethylene glycol + .5 M galactose + 18% Ficoll made in base medium) at 22 ± 2 °C. Embryos in the CON group were placed in < 1 µl V2 on to Cryotops, and after 35 s, vitrified by plunging directly into liquid nitrogen. Embryos cryopreserved via BLK were loaded into 0.25 mL straws, in 20 µl V2. Straws were preloaded with a column of 120 µl of 1 M galactose followed by an air bubble, then 20 µl V2 containing embryos followed by an air bubble, and another column of 60 µl of 1 M galactose, and then sealed with a plastic plug.

After 35 s in V2, embryos were vitrified by cooling for 2 min via contact of straw walls with columns drilled into an aluminum block immersed in liquid nitrogen, and then directly plunged into liquid nitrogen. Embryos were then stored in liquid nitrogen at -196 °C until warmed.

Conventional Freezing

Embryos cryopreserved by conventional slow freezing (SLF) were exposed to 1.36 M glycerol in modified Dulbecco's PBS + 0.4% BSA (PBS) for 10 min at 22 ± 2°C. Embryos were then loaded into 0.25 mL straws. Straws were loaded with a column of 80 µl of 1.36 M glycerol followed by an air bubble, 30 µl of 1.36 M glycerol containing embryos followed by an air bubble, and another column of 80 µl of 1.36 M glycerol, sealed with a plastic plug, and placed into a freezing machine. Straws were cooled to -6 °C at 4 °C per min, held at -6 °C for 5 min, seeded, held at -6 °C for an additional 10 min, and then cooled to -30 °C at 0.5 °C per min, plunged into liquid nitrogen, and stored in liquid nitrogen at -196 °C until thawed.

Warming/Thawing and Culture

After storage for at least 24 h in liquid nitrogen, embryos were warmed or thawed. Embryos vitrified using the CON method were removed from Cryotops by direct placement into a 200 µl drop of 1 M galactose in base media for 2 min. Those cryopreserved via BLK and SLF were warmed or thawed, respectively, by holding straws in air for 8 s and placing them in a water bath at 37 °C for 20 s. By shaking straws 4 times in a fashion similar to resetting a clinical thermometer,

BLK embryos were mixed with 1 M galactose in base media in the straw and held for 2 min whereas SLF embryos were expelled from straws, and glycerol was removed in a step-wise manner. Embryos were exposed to 0.8 M glycerol + 0.3 M sucrose for 6 min, 0.4 M glycerol + 0.3 M sucrose for 6 min, and 0.3 M sucrose for 6 min, followed by PBS for 2 min. After all embryos CON, BLK, and SLF were recovered, they were rinsed through holding chemically defined medium-2 (HCDM-2) for 2 min and cultured in chemically defined medium-2 (CDM-2; similar to SOF) for 24 h before being evaluated for survival. Embryos were evaluated morphologically for reformation of the blastocoele, expansion, and hatching, and scored for quality (1 = excellent; 2 = good; 3 = fair, 4 = poor; 5 = degenerate). Embryos of excellent, good, and fair quality were considered to have survived; poor quality and degenerate embryos were not.

Statistical Analysis

The percent survival for each treatment/replicate subclass was subjected to factorial analysis of variance (GLM, SAS) with factors treatments and replicates. Tukey's HSD test was used to determine statistical significance. Each treatment was replicated 7 times.

Results and Discussion

Post warming survival was greater ($P < 0.01$) for CON than BLK (85.9%, n = 118; 70.6%, n = 128, respectively); BLK was greater ($P < 0.01$) than SLF (56.1%, n = 131). Post warming survival did not differ (Fisher's Exact Test; $P > 0.1$) between bulls [Bull A = 64.5% (78/121), Bull B = 70.2% (59/84), Bull C = 67.4% (116/172)]. Two of nine replicates in which the control, Cryotop procedure failed (survival < 70%) were excluded from the results because survival rates were low for all treatments, indicating failure of the in vitro system.

It was not surprising that the Cryotop method, the "gold standard" for cryopreservation of human embryos, was superior to other cryopreservation techniques. Kuwayama et al. (2005), who developed the method, even cites 91% (58/64) survival of bovine oocytes (which are more difficult to cryopreserve than embryos; Prentice et al., 2011) after warming. This success can easily be explained by the reduced volume of vitrification solution associated with the Cryotop method, the greater surface to volume ratio of using < 1 µl versus the 20 µl with the BLK method allows for more rapid cooling, and therefore less damage to the embryos. The obvious flaw with the Cryotop method, however, is that a microscope and skilled technician are required to transfer embryos to a straw post warming before they can be transferred into a recipient, which is impractical for on-farm application.

Survival rates for embryos vitrified via BLK, although lower than CON, were acceptable. Large scale embryo transfer studies have been conducted yielding pregnancy rates for fresh IVF bovine embryos of 53.8% [(1220/2268); Hasler et al., 1995]. When using in vitro survival as an indicator of fetal development rate, BLK vitrification may be an improved method for direct transfer of IVP bovine embryos compared to SLF as it yielded greater post-warming survival than the current SLF method used for cryopreservation of bovine embryos.

Chapter 5

Experiment 3. Evaluation of fetal development rates of vitrified or slow frozen in vitro-produced bovine embryos following non-surgical transfer into recipients

Introduction

As stated in Experiments 1 and 2, in vitro-produced bovine embryos can be generated inexpensively, and are in demand to create pregnancies in both beef and dairy cattle, with fresh embryos resulting in acceptable pregnancy rates (53.8% (1,220/2,268); Hasler et al., 1995). Also, the number of transfers of in vitro-produced embryos has increased since 2000 (Stroud, 2010). Currently, however, efficacious cryopreservation methods that would enable direct transfer are not available for such embryos; only 7% of the in vitro-produced embryos transferred worldwide in 2009 were cryopreserved (Stroud, 2010).

Our laboratory has developed practical procedures by optimizing cryoprotectant concentrations, placing plastic straws in liquid nitrogen cooled air for vitrification, and optimizing timing of steps for vitrifying both early stage equine embryos (Eldridge-Panuska et al., 2005) and in vivo-produced bovine embryos (Campos et al., 2006); pregnancy rates have been excellent following embryo transfer directly from straws used for cryopreservation. Experiment 1 simplified the vitrification procedure by use of an aluminum block, making cooling more

consistent (Kruse and Seidel, 2010). Experiment 2 demonstrated superior post warming in vitro survival of embryos vitrified by contact of straw walls with holes in an aluminum block submerged in liquid nitrogen compared to conventional slow freezing methods (70.6% and 56.1%, respectively; $P < 0.01$). We hypothesized that the successful in vitro survival of these embryos would translate to acceptable pregnancy rates in recipients of such embryos. Therefore, the objective of this experiment was to compare pregnancy rates of recipients after direct transfer of in vitro-produced bovine embryos cryopreserved via 1) vitrification by contact of straw walls with holes in an aluminum block submerged in liquid nitrogen, or 2) conventional slow freezing.

Materials and Methods

In Vitro Embryo Production

In Experiment 3, expanded bovine blastocysts were produced by standard in vitro procedures developed by our laboratory (de la Torre-Sanchez et al., 2006). Briefly, cumulus-oocyte complexes were obtained by aspirating follicles from abattoir-derived ovaries of cull cows and matured for 23 h in vitro in chemically defined media containing follicle-stimulating and luteinizing hormones, 17-β estradiol, cystemine, and epidermal growth factor. After maturation, oocytes were fertilized by frozen/thawed semen from one of three bulls (one per replicate). Semen was centrifuged through a 45:90% Percoll gradient (2 mL each) in conical 15-mL test tubes for 20 min at 400 x gravity to separate primarily normal, motile sperm. Concentration was adjusted to 5×10^5 sperm/mL and

added to medium in wells containing oocytes for fertilization. After 18 h of coincubation of gametes, cumulus-oocyte complexes were vortexed to remove cumulus cells and dead sperm. Presumptive zygotes were cultured in chemically defined medium 1 (CDM-1) for 56 h at which time they were evaluated for cleavage. Those reaching at least the 8+ cell stage were then cultured in CDM-2 for 4 d at which time blastocysts were identified. Only d 7 expanded blastocysts of excellent quality with excellent ICM were cryopreserved. For every replicate cryopreserved, quality control embryos were warmed/thawed and cultured to ensure in vitro survival before being used for transfer. All embryos passed the warm/thaw and culture test as survival rates were no less than one standard deviation below the mean of survival rates reported in Experiment 2 where BLK = 70.6% and SLF = 56.1%. In fact, survival rates were greater than those reported in Experiment 2 with mean in vitro survival of quality control embryos exceeding 75%. Embryos were cryopreserved by vitrification using an aluminum block (VIT; n = 78), or by slow freezing (SLF; n = 78).

Vitrification

Standard vitrification procedures developed by our laboratory (Campos et al., 2006) were used with slight modifications. The base medium for all vitrification solutions was a specially formulated version of Syngro Holding Medium (Bioniche Life Sciences, Belleville, Ont) which had lowered concentrations of sodium and calcium (60 mM NaCl + 60 mM choline chloride and 0.5 mM calcium). Blastocysts were exposed to 5 M ethylene glycol made in base medium (V1) for 3 min at 22 ± 2 °C and moved in less than 1 µl of V1 into

20 µl of 6.5 M ethylene glycol + .5 M galactose + 18% Ficoll made in base medium (V2) at 22 ± 2 °C. VIT embryos (2 embryos per straw) were loaded into 0.25 mL straws, with a column of 120 µl of 1 M galactose followed by an air bubble, then 20 µl V2 containing the embryos followed by an air bubble, and another column of 60 µl of 1 M galactose, followed by sealing with a plastic plug. After 35 s in V2, embryos were vitrified by cooling for 2 min via contact of straw walls with columns drilled into an aluminum block immersed in liquid nitrogen, and then directly plunged into liquid nitrogen for storage at -196 °C until warmed.

Conventional Freezing

Embryos cryopreserved by conventional slow freezing (SLF) were exposed to 1.36 M glycerol in modified Dulbecco's PBS + 0.4% BSA (PBS) for 10 min at 22 ± 2 °C. SLF embryos were then loaded (2 embryos per straw) into 0.25 mL straws. Straws were loaded with a column of 80 µl of 1.36 M glycerol followed by an air bubble, 30 µl of 1.36 M glycerol containing embryos followed by an air bubble, and another column of 80 µl of 1.36 M glycerol, and then sealed with a plastic plug and placed into a freezing machine. Straws were cooled to -6 °C at 4 °C per min, held at -6 °C for 5 min, seeded, held at -6 °C for an additional 10 min, cooled to -30 °C at 0.5 °C per min, and then plunged into liquid nitrogen for storage at -196 °C until thawed.

Warming/Thawing

After storage for at least 24 h in liquid nitrogen, VIT and SLF embryos were warmed or thawed, respectively, by holding straws in air for 8 s and placing them in a water bath at 37°C for 20 s. By shaking straws four times in a fashion

similar to resetting a clinical thermometer, VIT embryos were mixed with 1 M galactose in base media in the straw and transferred to recipients within 2 to 5 min. SLF embryos were expelled from straws, and glycerol was removed in a step-wise manner. Embryos were exposed to 0.8 M glycerol + 0.3 M sucrose for 6 min, 0.4 M glycerol + 0.3 M sucrose for 6 min, and 0.3 M sucrose for 6 min, followed by PBS for 2 min. SLF embryos were then loaded into 0.25 mL straws previously loaded with PBS, followed by a column of air, PBS + embryos, air, PBS, and finally air, and transferred to recipients within 2 to 5 min of loading into the 0.25 mL straw. Embryos in SLF group were evaluated upon thawing, but were transferred regardless of quality to prevent bias, as those in the VIT group were not evaluated between warming and transfer.

Embryo Transfer and Recipients

All animals were handled in accordance with Colorado State University IACUC guidelines (Practical vitrification of bovine embryos; protocol # 10-1975A). Crossbred, non-lactating beef cows purchased at the local livestock auction market and culled for unknown reasons, but with normal-appearing reproductive tracts served as recipients. Criteria for retaining cattle included being no more than 30 d pregnant (aborted with prostaglandin F2α), having a fully developed reproductive tract free of adhesions and abnormalities, having sound mouths to maintain body condition, sound feet and legs, body condition score between 4-7 (on a scale of 1-9; where 1 = emaciated; 9 = obese), and a good temperament. Cows were maintained in 10 head pens on a 60:40 corn stocks: corn silage diet, and synchronized with the double shot prostaglandin F2α protocol. Embryos

were transferred nonsurgically by experienced technicians. Recipients were d 7 ± 0.5 of the estrous cycle, and each received two embryos into the uterine horn ipsilateral to the CL following epidural anesthetic using 5 mL of 1% Lidocaine solution. Cattle were observed for signs of estrus every 12 h following embryo transfer by visual detection aided by Estrotect Alert patches. Pregnancy diagnosis was performed at d 37 ± 2 via ultrasonography. Only normal appearing fetuses with a heartbeat were considered successful fetal development. Pregnancies were terminated with i.m. injection of 25 mg prostaglandin F2α (Lutalyse; Pfizer Animal Health, New York City, NY, USA).

Statistical Analysis

Fisher's Exact Test was applied in order to test the associations between cryopreservation method, embryo quality at time of transfer, sire, and transfer quality. The 10% ($P < 0.1$) probability level was the threshold used for statistical significance.

Results and Discussion

Survival rate per embryo (normal fetus with heartbeat) did not differ (Fisher's Exact Test; $P > 0.10$) between cryopreservation methods (VIT = 14.1% (11/78); SLF = 16.7% (13/78); 9 of the 15 pregnant cows carried twins). Survival rate per embryo by embryo quality at time of transfer (only able to be evaluated in SLF treatment) were as follows: Q#1 = 22.2% (6/27), Q#2 = 15.2 % (5/33), Q#3 = 15.4% (2/13), Q#4 = 0% (0/4), Q#5 = 0% (0/1), and did not differ due to quality ($P > 0.10$). Pregnancy per embryo did not differ (Fisher's Exact Test; P >

0.10) between sire [Bull A = 15.9% (7/44), Bull B = 15.8% (12/76), Bull C = 13.9% (5/36]). There were no bull by TRT interactions. Transfer quality did not affect pregnancy rate (P > 0.10; Q#1 = 17.6% (19/108), Q#2 = 17.9% (5/28)). Although not statistically significant, no transfers of quality 3 resulted in pregnancy (Q#3 = 0% (0/20). There were no transfer quality by TRT interactions.

Return to estrus of recipients was quantified in the following manner: 1 = abnormally short estrus interval, < 17 d following last estrus; 2 = estrus at normal interval, 17-23 d following last estrus; 3 = abnormally long estrus interval, 24-37 d following last estrus; 4 = pregnant, not detected in estrus; 5 = not pregnant, not detected in estrus. Return to estrus by treatment is as follows in **Table 5.1**:

Table 5.1. Return to estrus by TRT where 1 = abnormally short estrus interval, 2 = estrus at normal interval, 3 = abnormally long estrus interval, 4 = pregnant, not detected in estrus, 5 = not pregnant, not detected in estrus.

Estrous Behavior X Cryopreservation TRT	**1**	**2**	**3**	**4**	**5**	**Total**
VIT (n, %**)**	**1**, *3%*	**14**, *35%*	**8***, 20%*	**6**, *15%*	**11**, *28%*	**40**
SLF (n, %**)**	**1**, *3%*	**18**, *45%*	**7**, *18%*	**9**, *23%*	**5**, *13%*	**40**

Estrous Behavior did not differ between TRT (Fisher's Exact Test; P > 0.10).

Females that returned to estrus early were excluded from results. Estrous behavior did not differ (Fisher's Exact Test; P > 0.10) between cryopreservation

methods. There were no estrous behavior by TRT interactions. Embryonic loss was the likely explanation for recipients that came into estrus 24-37 d after last exhibiting estrus; 19.2% of all recipients were in this category.

Although pregnancy rates of recipients receiving in vitro-produced embryos were poor, other investigators have experienced similar results with cryopreserved IVP bovine embryos (13% and 25%, Tachikawa et al., 1993; 44% and 23%, Agca et al., 1998a; 38% and 26%, Agca et al., 1998b; 9% and 0%, Donnay et al., 1998; 22% Pugh et al., 2000; 6.5%, Al-Katanani et al., 2002; 30%, Nedambale et al., 2004; 24% and 14% Wurth et al., 1994, 24% and 14%, Lim et al., 2008); none of these studies had more than a few dozen embryos per treatment.

It is important to note that SLF embryos were transferred regardless of quality upon thawing to test the hypothesis that rapid cooling induced by vitrification would be a superior method of cryopreserving embryos. If we unfairly eliminated those that did not survive cryopreservation in only the conventionally frozen group, it would not have been a fair comparison of the cryopreservation methods. Also, because we were dealing with in vitro-produced embryos, (which are cryopreserved less successfully than in vivo-produced embryos; Hasler, 1998), we chose to use glycerol as a cryoprotectant instead of ethylene glycol because the removal of glycerol in a three step manner is less stressful to embryos than a single in-straw dilution of ethylene glycol.

It should also be mentioned that embryo transfers occurred between October 15 and December 21, often in inclement, very cold weather. Furthermore, recipients were on a low plane of nutrition (60:40 corn stalks: corn silage ration) during that time, losing an average of 79 lbs. over the 3+ month period. Recipient cows were purchased from a local livestock auction market, culled for unknown reasons, but with normal-appearing reproductive tracts. Criteria for retaining cattle included being no more than 30 d pregnant (aborted with PGF2α), having a fully developed reproductive tract free of adhesions and abnormalities, having sound mouths to maintain body condition, sound feet and legs, body condition score between 4-7 (on a scale of 1-9; where 1 = emaciated; 9 = obese), and a good temperament. Of the cows purchased, < 30% were selected to serve as recipients. Still, there were a disproportionately high number of recipients pregnant with twins (9 of the 15 pregnant cows carried twins). Given the overall fetal development rate per embryo of 15.3%, we would expect 2.3% of the recipients to be pregnant with twins, but in this case, 11.5% (9/78) of the cows carried twins. This leads us to believe the uteri of some recipients within this particular population of cattle were not conducive to maintenance of pregnancy for unknown reasons. Further supporting this argument is that for every replicate cryopreserved, quality control embryos cryopreserved identically to those to be transferred into recipients were warmed/thawed and cultured to ensure in vitro survival before being used for transfer. All replicates passed the warm/thaw and culture test as survival rates were no less than one standard deviation below the mean of survival rates

reported in Experiment 2 in which BLK = 70.6% and SLF = 56.1%. Survival rates were actually greater than those reported in Experiment 2 with mean in vitro survival of quality control embryos (cryopreserved by both VIT and SLF) exceeding 75%.

Although no improvement in pregnancy rate by cryopreserving embryos via vitrification was demonstrated by this study, the technology offers benefits in terms of reduced time requirements and inexpensive equipment and should still be superior to freezing, in theory. A recent study by Lim et al. (2008) demonstrated higher pregnancy rates at d 35 (31.9% versus 22.9%) for in vitro-produced embryos vitrified in medium supplemented with 0.4% lipid-rich BSA in which sodium chloride was totally replaced with choline chloride compared to embryos vitrified in standard medium. Therefore, modification of the freezing medium used in this experiment could be a method of improving post-transfer pregnancy rates of in vitro-produced embryos.

Chapter 6

Experiment 4. Evaluation of fetal development rates of vitrified or slow frozen in vivo-produced bovine embryos following non-surgical transfer into recipients

Introduction

The first report of a mammalian embryo successfully frozen was in 1972 (Whittingham et al.) in the mouse. Not long after, the first live calf was born from embryo transfer of a frozen/thawed bovine embryo (Wilmut and Rowson, 1973). Because conventional slow freezing was developed first and produced acceptable pregnancy rates after embryo transfer (now 80-90% of non-frozen controls; Hasler, 2001), it became the industry standard for bovine embryo cryopreservation. Although pregnancy rates are quite good from transfer of embryos cryopreserved via conventional freezing, the technology presents some challenges: 1) freezing equipment is costly, 2) protocols typically take over an hour, 3) ice crystal formation can damage embryos, and 4) technical difficulties can occur with seeding.

Vitrification was developed 13 years later (Rall and Fahy, 1985) with the primary advantage over freezing being cryopreservation without forming damaging ice crystals. Other benefits include minimal time and cost associated with this technique. Hundreds of vitrification studies have been published (Vajta

and Nagy, 2006), and several vitrification protocols exist (Martinez et al., 1998; Park et al., 1999; Kaidi et al., 1999; Nguygen et al., 2000; Kaidi et al., 2000; Martinez et al., 2002; Seidel and Walker, 2006), but adoption of these methods has not occurred in commercial bovine embryo transfer practices (American Embryo Transfer Association, 2010).

In Experiment 3, pregnancy rates of recipients of in vitro-produced embryos were poor and fetal survival rates did not differ between those cryopreserved via vitrification or conventional freezing (14.1% and 16.7%, respectively; $P > 0.10$). However, a very convincing study was conducted on a large scale by van Wagtendonk de Leeuw et al. (1997) in which 728 in vivo-produced bovine embryos were cryopreserved in 0.25 ml straws either by vitrification or conventional slow freezing, and resulting pregnancy rates were acceptable and did not differ between cryopreservation methods (44.5% and 45.1%, respectively). Also, the survival rates of embryos to fetuses in Experiment 3 were too low for a meaningful test of the main hypothesis. Therefore, the objective of Experiment 4 was to determine if the cryopreservation methods used in Experiment 3 were inferior, and if vitrification, in fact, was sufficiently effective to reduce time and expense for in vivo-produced embryos.

Materials and Methods

Cattle

All animals were handled in accordance with Colorado State University IACUC guidelines (Practical vitrification of bovine embryos; protocol # 10-1975A).

In Vivo Embryo Recovery

In Experiment 4, crossbred, nonlactating beef cows were enrolled in 1 of 3 superovulation treatments 8 to12 d after being observed in standing estrus and confirmation of presence of a CL as determined by rectal palpation. Donors in all treatments were superovulated via administration of follicle stimulating hormone (FSH) i.m. (Folltropin®-V; Bioniche Life Sciences, Belleville, Ont), and luteolysis was induced by i.m. injection of prostaglandin F2α followed by estrus detection at 12 h intervals.

In TRT 1, donors were given two injections for a total of 280 mg FSH reconstituted with a slow release formula (a proprietary diluent provided by Bioniche Animal Health, Inc.): 188 mg FSH at 0 h and 92 mg FSH at 48 h, where 0 h equals the start of superovulation protocol. The second dose of FSH was accompanied by a single dose of 37.5 mg prostaglandin F2α. In TRT 2, donors were given two injections for a total of 140 mg FSH reconstituted with a slow release formula; 94 mg FSH at 0 h and 46mg FSH at 48 h, where 0 h equals the start of superovulation protocol. A single dose of 37.5 mg prostaglandin F2α was given with the second dose of FSH. In TRT 3, donors were given eight injections for a total of 280 mg FSH reconstituted with diluent (Folltropin®-V Diluent; Bioniche Life Sciences, Belleville, Ont); 60 mg at 0 and 12 h, 40 mg at 24 and 36 h, and 20 mg at 48, 60, 72, and 84 h. Prostaglandin F2α was administered twice; 25 mg at 60 h and 12.5 mg at 72 h, where 0 h equals the start of superovulation protocol. Donors in all treatments were artificially inseminated at 12 and 24 h

after standing estrus with semen from 1 of 3 bulls, the same sires used for in vitro fertilization of the IVP embryos in Experiment 3.

Embryos were recovered nonsurgically 7 to 7.5 d post estrus and evaluated microscopically to determine quality and stage. Compact morulae, blastocysts, and expanded blastocysts of quality #1 or #2 per IETS standards (scale 1-5; 1 = excellent, 5 = degenerate) were washed through several changes of Syngro Holding Medium (Bioniche Life Sciences, Belleville, Ont) and kept at 22 ± 2 °C until cryopreserved via vitrification (VIT; n = 46) or slow freezing (SLF; n = 44).

Procedures for vitrification, conventional freezing, warming/thawing, and transfer of embryos in to recipients were identical to those described in Experiment 3.

Statistical Analysis

Fisher's Exact Test was applied in order to test the associations between cryopreservation method, embryo quality at time of transfer, sire, embryo transfer technician, and transfer quality. The 10% ($P < 0.1$) probability level was the threshold used for statistical significance.

Results and Discussion

In Experiment 4, survival rate per embryo (normal fetus with heartbeat) did not differ (Fisher's Exact Test; P > 0.10) between cryopreservation methods (VIT = 45.7% (21/46); SLF = 38.6% (17/44); 17 of the 21 pregnant cows carried twins). Survival rate per embryo by embryo quality at time of transfer (only able to be evaluated in SLF treatment) were as follows: Q#1 = 42.8% (6/14), Q#2 = 39.3 % (11/28), Q#3 = 0.0% (0/2), and did not differ due to quality (P > 0.10). Fetal survival per embryo did not differ (Fisher's Exact Test; P > 0.10) between embryo quality at time of cryopreservation [Q#1 = 46.6% (27/58), Q#2 = 34.3 % (11/32)]; sire [Bull A = 38.2% (13/34), Bull B = 50.0% (6/12), Bull C = 43.2% (19/44)]; or ET technician [GF = 53.8% (14/26), ZB = 37.5% (24/64)]. Transfer quality did not affect pregnancy rate [Q#1 = 42.4% (28/66), Q#2 = 50.0% (8/16), Q#3 = 25% (1/4)]. There were no embryo quality at time of cryopreservation by TRT interactions, sire by TRT interactions, ET technician by TRT interactions, or transfer quality by TRT interactions.

Return to estrus of recipients was quantified in the following manner: 1 = abnormally short estrus interval, < 17 d following last estrus; 2 = estrus at normal interval, 17-23 d following last estrus; 3 = abnormally long estrus interval, 24-37 d following last estrus; 4 = pregnant, not detected in estrus; 5 = not pregnant, not detected in estrus. Return to estrus by treatment is as follows in **Table 6.1**:

Table 6.1. Return to estrus by TRT where 1 = abnormally short estrus interval, 2 = estrus at normal interval, 3 = abnormally long estrus interval, 4 = pregnant, not detected in estrus, 5 = not pregnant, not detected in estrus.

Estrous Behavior X Cryopreservation TRT	1	2	3	4	5	Total
VIT (n, %**)**	**0**, *0%*	**5**, *22%*	**3***, 13%*	**11**, *48%*	**4**, *17%*	**23**
SLF (n, %**)**	**0**, *0%*	**8**, *36%*	**1**, *5%*	**10**, *45%*	**3**, *14%*	**22**

Estrous Behavior did not differ between TRT (Fisher's Exact Test; P > 0.10).

Estrous behavior did not differ (Fisher's Exact Test; P > 0.10) between cryopreservation methods. There were no estrous behavior by TRT interactions. Embryonic loss was the likely explanation for recipients that came into estrus 24-37 d after last exhibiting estrus; 8.9% of all recipients were in this category.

Mean numbers of embryos of transferable quality recovered per donor by superovulation TRT were as follows:

Table 6.2. Transferable embryos per donor by superovulation TRT; where n = number of cows superovulation TRT was applied to, Embryos/Donor = number of embryos recovered per donor flushed, S.D. = standard deviation from mean number of embryos recovered

Superovulation TRT	n	Embryos/Donor	S.D.
1 280 mg FSH - 2 injections; 37.5 mg PGF – 1 injection	12	2.3	2.96
2 140 mg FSH - 2 injections; 37.5 mg PGF – 1 injection	4	8.0	8.83
3 280 mg FSH - 8 injections; 37.5 mg PGF – 2 injections	6	6.5	5.17

The overall pregnancy rate of 42.2% is comparable with that of other studies after transfer of cryopreserved in vivo-produced embryos (overall = 44.2%, vitrified = 36.1%, slow frozen = 59%; van Wagtendonk-De Leeuw et al., 1995; overall = 44.6%, vitrified = 44.5%, slow frozen = 45.1%; van Wagtendonk-De Leeuw et al., 1997). The aforementioned studies are the only ones in the literature which compare pregnancy rates of 100+ recipients after transfer of in vivo-produced embryos cryopreserved via vitrification.

It is important to note that SLF embryos were transferred regardless of quality upon thawing. This is not a practice that would normally be done in a clinical setting when glycerol is used as the cryoprotectant, which may explain why those pregnancy rates are poorer than industry averages. However, we were seeking to test the hypothesis that rapid cooling induced by vitrification would be a superior method of cryopreserving embryos. If we unfairly eliminated those that did not survive cryopreservation in only the conventionally frozen group, it would not have been a fair comparison of the technologies. Also, the use of glycerol as a cryoprotectant instead of ethylene glycol is no longer common practice in commercial embryo transfer; less than 2% of embryos were cryopreserved with glycerol in 2009 (American Embryo Transfer Association, 2010). However, removal of glycerol in a three step manner is a less stressful method to embryos when removing cryoprotectant than with the single in-utero dilution of ethylene glycol, especially for in vitro-produced embryos. Because we used glycerol for cryopreservation of the in vitro-produced embryos in Experiment 3, we wanted to keep the comparison consistent.

Superovulation of donors was not designed as an experiment. We began by using FSH reconstituted with a slow release formula (SRF; not yet commercially available) because injections were required only 2 times as opposed to 8. Upon use of 280 mg FSH diluted with SRF (TRT 1) we noticed ovaries of those donors seemed to be over-stimulated; 6 of 12 donors had 20+ CL in total, and donors with more than 13 CL did not produce more than 2 useable embryos. Therefore, we dropped the dose to half, while still only having to administer 2 injections with the SRF formula (TRT 2) and noticed a better response. Even so, toward the end of the experiment, we were concerned about collecting enough embryos to complete the experiment in a timely manner, so we decided to use a proven method that had worked very successfully for our lab in the past (TRT 3).

Also, embryo transfers occurred between December 12 and January 30, often in inclement, very cold weather. Recipient cows were purchased from a local livestock auction market, culled for unknown reasons, but with normal-appearing reproductive tracts. Criteria for retaining cattle included being no more than 30 d pregnant (aborted with prostaglandin F2α), having a fully developed reproductive tract free of adhesions and abnormalities, having sound mouths to maintain body condition, sound feet and legs, body condition score between 4-7 (on a scale of 1-9; where 1 = emaciated; 9 = obese), and a good temperament. Of the cows purchased, < 30% were selected to serve as recipients. Even so, there were a disproportionately high number of recipients pregnant with twins (17 of the 21 pregnant cows carried twins). Given the overall survival rate per

embryo of 42.2%, we would expect 17.8% of the recipients to be pregnant with twins, but in this case, 37.8% (17/45) of the pregnant cows carried twins. This leads us to believe the uteri of some recipients within this particular population of cattle were not conducive to maintenance of pregnancy for unknown reasons.

Although no improvement in pregnancy rate by cryopreserving embryos via vitrification was demonstrated by this study, the technology offers benefits in terms of reduced time requirements and inexpensive equipment. However, the advantage of vitrification is most obvious when cryopreserving a small number of embryos, as vitrification occurs in a few minutes, whereas slow freezing protocols take over 1 h.

Chapter 7

Conclusions

In Experiment 1, although post warming survival did not differ (P > 0.10) between treatments (AIR x CON = 42.0%; AIR x LOW = 26.8%; BLK x CON = 21.8%; BLK x LOW = 24.5%), we recommend use of base media with lowered sodium and calcium concentrations (LOW), which should have less chance of both sodium and calcium toxicity, and could deter apoptosis. Vitrification via contact of straw walls with an aluminum block cooled in liquid nitrogen (BLK) is both easier to use and more consistent than placing straws in liquid nitrogen-cooled air. Low survival rates in this experiment led us to conclude that stage and quality of blastocyst are extremely important in their ability to survive cryopreservation; oocytes derived from ovaries of cull cows result in more robust blastocysts than those derived from feedlot heifers (Barceló-Fimbres et al, 2011), and water quality is key for successful blastocyst production. Our later experiments were limited to the use of only excellent quality expanded blastocysts derived from cull cows.

In Experiment 2, post warming survival was greater ($P < 0.01$) for embryos vitrified on Cryotops (CON) than BLK (85.9%, n = 118; 70.6%, n = 128, respectively); BLK was greater ($P < 0.01$) than conventional slow freezing; SLF (56.1%, n = 131). Although the Cryotop method was superior, its use requires a microscope and skilled technician to transfer embryos to a straw before they can be transferred into a recipient, which is impractical for on farm application. Survival rates for embryos vitrified via BLK, although lower than CON, were acceptable; therefore, BLK vitrification may be an appropriate method for direct transfer, which yielded greater post-warming survival than the current SLF method used for cryopreservation of in vitro-produced bovine embryos.

In Experiments 3 and 4, survival rates of embryos to fetuses following transfer of in vitro- or in vivo-produced embryos and returns to estrus did not differ between cryopreservation methods (Exp 3: VIT = 14.1%, SLF = 16.7%, with 9 of the 15 pregnant cows carrying twins; Exp 4: VIT = 45.7%, SLF = 38.6%, with 17 of the 21 pregnant cows carrying twins); results were similar to those of other investigators after transfer of cryopreserved bovine embryos. Although no improvement in pregnancy rate by cryopreserving embryos via vitrification was demonstrated by these studies, the technology offers benefits in terms of reduced time requirements and inexpensive equipment. Even so, the advantage of vitrification is most obvious when cryopreserving a small number of embryos as vitrification occurs in a few minutes, whereas slow freezing protocols take over 1 h.

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