Consequences of transfer of an in vitro-produced embryo for the dam and resultant calf¹

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ABSTRACT

No reports exist on consequences of in vitro production (IVP) of embryos for the postnatal development of the calf or on postparturient function of the dam of the calf. Three hypotheses were evaluated: calves born as a result of transfer of an IVP embryo have reduced neonatal survival and altered postnatal growth, fertility, and milk yield compared with artificial insemination (AI) calves; cows giving birth to IVP calves have lower milk yield and fertility and higher incidence of postparturient disease than cows giving birth to AI calves; and the medium used for IVP affects the incidence of developmental abnormalities. In the first experiment, calves were produced by AI using conventional semen or by embryo transfer (ET) using a fresh or vitrified embryo produced in vitro with X-sorted semen. Gestation length was longer for cows receiving a vitrified embryo than for cows receiving a fresh embryo or AI. The percentage of dams experiencing calving difficulty was higher for ET than AI. We observed a tendency for incidence of retained placenta to be higher for ET than AI but found no significant effect of treatment on incidence of prolapse or metritis, pregnancy rate at first service, services per conception, or any measured characteristic of milk production in the subsequent lactation. Among Holstein heifers produced by AI or ET, treatment had no effect on birth weight but the variance tended to be greater in the ET groups. More Holstein heifer calves tended to be born dead, died, or were euthanized within the first 20 d of life for the ET groups than for AI. Similarly, the proportion of Holstein heifer calves that either died or were culled for poor health after 20 d of age was greater for the ET groups than for AI. We observed no effect of ET

compared with AI on age at first service or on the percentage of heifers pregnant at first service, calf growth, or milk yield or composition in the first 120 d in milk of the first lactation. In a second experiment, embryos were produced using 1 of 2 culture media: synthetic oviductal fluid–bovine embryo 1 (SOF-BE1) or Block-Bonilla-Hansen 7 (BBH7). We detected no difference between cows receiving an SOF-BE1 or BBH7 embryo in gestation length, the percentage of cows in which parturition was induced, or the percentage of cows that experienced calving difficulty, retained placenta, prolapse, or metritis. Among Holstein heifers, birth weight was higher for BBH7 calves than for SOF-BE1 calves. Treatment had no significant effect on calf death. Results indicate that calves born as a result of IVP-ET are more likely to experience alterations in birth weight and increased death in early life but that there were few consequences to the dam of carrying a fetus derived by IVP-ET.

Key words: embryo transfer, in vitro fertilization, calf mortality, reproduction

INTRODUCTION

In vitro production of embryos (IVP) is a technology that can be used to increase genetic selection (Moghaddaszadeh-Ahrabi et al., 2012; Weigel et al., 2012), utilize sex-sorted semen (Xu et al., 2006; Rasmussen et al., 2013), and improve fertility during heat stress (Block et al., 2010; Stewart et al., 2011; Vasconcelos et al., 2011) and in repeat-breeder females (Son et al., 2007; Block et al., 2010; Canu et al., 2010). The opportunities created by IVP have not been fully realized, however, because the IVP embryo is aberrant in certain respects compared with embryos produced in vivo. Molecular differences in the function of embryonic blastomeres, as illustrated by alterations in DNA methylation (Niemann et al., 2010), the transcriptome (Driver et al., 2012), and lipidome (Sudano et al., 2012), result in an embryo with reduced capacity for establishment of pregnancy after transfer to recipient females (Farin et al., 1999; Pontes et al., 2009; Siqueira et al., 2009). In vitro production has longer-term conse-

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quences, too, with reports of higher pregnancy losses in the late embryonic and fetal period (Agca et al., 1998), abnormal placental development (van Wagtenonk-de Leeuw et al., 1998; Bertolini et al., 2004; Miles et al., 2004, 2005), heavier fetal (Bertolini et al., 2004; Miles et al., 2004) and birth weights (Behboodi et al., 1995; Kruip and Den Haas, 1997; van Wagtenonk-de Leeuw et al., 1998; Numabe et al., 2000; Breukelman et al., 2005), and increased rates of dystocia (Behboodi et al., 1995; van Wagtenonk-de Leeuw et al., 1998), congenital abnormalities (Wagtenonkde Leeuw et al., 1998), stillbirths, and neonatal mortality (Kruip and Den Haas, 1997; van Wagtenonk-de Leeuw et al., 1998; Numabe et al., 2000). Also, the sex ratio at birth can be skewed toward the male (van Wagtenonk-de Leeuw et al., 1998; Block et al., 2003). Increased frequency of developmental defects in IVP-derived offspring has not always been observed (Jiang et al., 2011; Rasmussen et al., 2013). Discrepancies in effects of IVP on the incidence of developmental defects could be due to differences in the culture systems used to produce embryos in vitro (Fischer-Brown et al., 2005) or be an artifact of small sample size.

There are no reports on consequences of IVP for the postnatal development of the calf or on postparturient function of the dam of the calf. It is reasonable to hypothesize that milk yield of cows that calved as a result of transfer of an IVP embryo would be altered because the fetus can affect subsequent milk yield (Cue and Hayes, 1985; Moya et al., 1989; Guilbault et al., 1990). Large birth weights and associated dystocia could also compromise the subsequent health of the dam because of the relationship between dystocia and subsequent reproductive function and milk yield (Zaborski et al., 2009).

Three hypotheses were evaluated in the current study. The first was that animals born as a result of IVP have reduced neonatal survival and altered postnatal growth, fertility, and milk yield compared with animals born as a result of AI. The second was that cows giving birth to calves produced as a result of IVP would have lower milk yield and fertility and higher incidence of postparturient disease than cows giving birth to calves born as a result of AI. Finally, we hypothesized that the culture medium used to produce embryos in vitro would affect the incidence of developmental abnormalities. The media assessed were synthetic oviductal fluid-bovine embryo 1 (**SOF-BE1**), a serum-free medium that includes BSA as the only protein source (Fields et al., 2011), and Block-Bonilla-Hansen 7 (**BBH7**; Cooley Biotech LLC, Gainesville, FL), a proprietary medium reported to increase the percentage of embryos that develop to the blastocyst stage and improve embryo survival after cryopreservation (Block et al., 2010).

MATERIALS AND METHODS

Experiment 1: Characteristics of Offspring and Dams in an IVP Program Compared with an AI Program

This study examined the characteristics of calves produced as a result of embryo transfer (**ET**) using a fresh IVP embryo (**ET-F**), ET using a vitrified IVP embryo (**ET-V**), or AI. In addition, health traits, reproductive function, and milk yield of their dams were determined. The experiment was conducted at the University of Florida Dairy Unit (Hague, FL; 29.77904 N, 82.48001 W). Data from 426 cows and their calves were analyzed. Data for pregnancy diagnoses at d 32 and 76 of gestation for 386 of the cows have been reported previously (Block et al., 2010). Details of reproductive management and embryo production for all 426 cows are provided in Block et al. (2010). For the ET groups, embryos were produced in vitro using BBH7 culture medium (Block et al., 2010). Data on cows were analyzed from d 76 of pregnancy through 210 d of the subsequent lactation, whereas data on calves were analyzed from birth until 120 d of the first lactation.

Embryos were produced using 2 bulls who together had an average PTA for various traits as follows: \$209 lifetime net merit (range 167 to 251), 12 kg of milk yield (−93 to 117), 3.9 yr of productive life (3.8 to 4), 7.2% sire calving ease (6.0–8.4%), 6.8% daughter calving ease (6.0 to 7.6%), 8.7% sire stillbirth (8.6 to 8.8%), 7.7% daughter stillbirth (6.6 to 8.8%), and 1.6% daughter pregnancy rate (0.8 to 2.3%). For AI, a total of 8 bulls were used, with average PTA as follows: \$257 lifetime net merit (range 15 to 447), 352 kg of milk yield (−147 to 651), 2.0 yr of productive life (−1.3 to 3.5), 6.3% sire calving ease (5.2 to 7.6%), 6.7% daughter calving ease (6.0 to 8.6%), 7.5% sire stillbirth (6.1 to 10.8%), 7.0% daughter stillbirth (6.6 to 10.8%), and 0.8% daughter pregnancy rate (−1.3 to 3.7%).

Dams. Holstein cows that were pregnant as a result of ET or AI were housed in sand-bedded freestall barns equipped with fans and sprinklers that were programmed to run when dry bulb temperatures exceeded 21.0°C. Cows were milked and fed a TMR twice daily. Cows were dried off 46 d before expected calving by cessation of milking and intramammary infusion of each quarter with antibiotic (Quartermaster, Pfizer Animal Health, Kalamazoo, MI). Parturition occurred spontaneously or, for animals that did not calve by 280 d of gestation, by induction via i.m. injection of 20 mg of dexamethasone (Aspen Veterinary Resources, Liberty, MO) and 25 mg of PGF_{2α} (Lutalyse, Pfizer Animal Health). Occurrence of one or more calving difficulties (needed assistance, slight problem, rear presentation, considerable force, extremely difficult and died) was re-

corded, as was occurrence of retained placenta, uterine prolapse, and metritis.

A timed AI (TAI) program was initiated at 38 to 44 DIM, when cows received a presynchronization protocol consisting of 2 injections of 25 mg of PGF_{2α}, i.m., 14 d apart. Twelve days after the second PGF_{2α} injection, cows were examined by rectal palpation for the presence of a corpus luteum (CL). If a CL was present, an Ovsynch-56 timed ovulation protocol (Brusveen et al., 2008) was initiated [100 µg of GnRH (Cystorelin, Merial, Duluth, GA), i.m., on d -10 relative to insemination; 25 mg of PGF_{2α}, i.m., on d -3; and 100 µg of GnRH, i.m., at 56 h after PGF_{2α}]. Cows were inseminated using a single straw of unsorted Holstein semen 16 h after the second GnRH injection. If a CL was not present, the Ovsynch-56 protocol was postponed for at least 1 wk until a CL was detected. Pregnancy diagnosis was performed by ultrasound on 32, 46, and 76 d after AI. Cows that were diagnosed as nonpregnant were subjected to additional rounds of Ovsynch-56 until pregnancy was confirmed.

Data on milk production were obtained from twice-daily measurements of milk yield and composition (protein and fat) using the Afimilk milk recording system (S.A.E. Afikim, Kibbutz Afikim, Israel). The relationship between protein and fat measurements using the Afimilk system and measurements from DHI has been reported previously (Kanyiamattam et al., 2010).

Calves. At birth, calf weight was recorded along with breed, sex, and occurrence of visual abnormalities. Calves were fed with colostrum within 1 h of birth and then housed individually in calf hutches under a pole-barn shade structure until approximately 60 d of age when calves were weaned and grouped in pens of 8 to 10 animals. Calves were transferred to larger pens in groups of 18 to 20 heifers at approximately 130 d of age.

A TAI program based on the 5-d Co-Synch + controlled internal drug release (CIDR) protocol (Rabagliano et al., 2010) was initiated when a heifer was at least 1.27 m tall, weighed more than 363 kg, and had symptoms of estrous cyclicity based on rectal palpation. The protocol consisted of insertion of a CIDR (Pfizer Animal Health) from d -8 to -3 relative to AI; 25 mg of PGF_{2α}, i.m., on d -3 and d -2; and 100 µg of GnRH, i.m., on d 0. Each heifer was AI on d 0 with a single straw of unsexed semen. Pregnancy status was determined by ultrasound between 32 to 33 d after insemination and reconfirmed by rectal palpation at 45 and 75 d. Nonpregnant heifers were re-enrolled in the TAI protocol. Heifers that were not pregnant after 5 breedings were re-evaluated for further breeding or culling. Following calving, heifers were managed using similar procedures as for their dams.

Body weight and height at the withers was measured at monthly intervals from 3 mo until 15 mo of age. In addition, data were collected on whether a calf died or was euthanized in the first 20 d of life, died or was culled for poor health after 20 d of life, or was culled after 20 d of life for poor growth or reproduction.

Experiment 2: Outcome of ET as Modified by Culture Medium

Experiment 2 was conducted to determine whether the type of culture medium used to produce embryos in vitro affected pregnancy rate or characteristics of the calves born as a result of ET. Embryos were produced by in vitro fertilization using X-sorted semen and were cultured in 1 of 2 culture media: SOF-BE1 (Fields et al., 2011) or BBH7 (Block et al., 2010). Blastocyst-stage embryos were then transferred to lactating recipients and subsequent pregnancy and calving rates as well as calf characteristics determined.

Embryo Production. Holstein cumulus-oocyte complexes (COC) harvested from abattoir ovaries were purchased from Sexing Technologies (Laceyville, PA) and shipped overnight in portable incubators (Cryologic Biotherm, Mulgrave, VIC, Australia) containing groups of 50 COC per 1.5 mL of maturation medium tube. The maturation medium was proprietary. After 22 to 24 h of maturation, COC were washed in HEPES-TALP (Tyrode's + BSA + lactate + pyruvate; Parrish et al., 1986) and placed in fertilization microdrops covered in mineral oil. Each droplet was approximately 80 µL and consisted of 50 µL of synthetic oviduct fluid-fertilization (SOF-FERT) (Sakatani et al., 2012), 1×10^6 /mL of X-sorted sperm (added in 20 µL of SOF-FERT), 3 µL of PHE solution [0.5 mM penicillamine, 0.25 mM hypotaurine, and 67.5 µM epinephrine, where the epinephrine was prepared as a 250 µM stock in a solution of 1 mg/mL sodium metabisulfite dissolved in a 1:649 (vol/vol) mixture of 98% (wt/wt) sodium lactate syrup in water], and approximately 30 COC (transferred in ~8 µL of HEPES-TALP; Parrish et al., 1986). Fertilization was allowed to proceed for 10 h in an incubator at 38.5°C and a humidified atmosphere of 5% (vol/vol) CO₂. Subsequently, presumptive zygotes were divided in groups of 30 in 50-µL microdrops of either SOF-BE1 or BBH7 and cultured at 38.5°C in a humidified atmosphere of 5% (vol/vol) CO₂, 5% (vol/vol) O₂, and the balance N₂. The proportion of oocytes that cleaved was recorded on d 3 after insemination, and the proportion of oocytes that developed to the blastocyst stage, including nonexpanded, expanded, hatching, and hatched, was determined at 7 d after insemination. Grade I (Robertson and Nelson, 1998) expanded blastocysts were harvested and loaded into

0.25-mL straws (AgTech, Manhattan, KS) containing holding medium [HEPES-TALP containing 5% (vol/vol) fetal calf serum (Atlanta Biologicals, Lawrenceville, GA) and 50 μ M DL-dithiothreitol] for embryo transfer.

Embryos were produced on 15 occasions (i.e., replicates). A total of 4 bulls were used to produce embryos; semen from a single bull was used for each replicate and the 4 bulls were rotated in sequence between replicates. The average PTA was as follows: \$308 lifetime net merit (range -59 to 469), 79 kg of milk yield (-9 to 287), 3.8 yr of productive life (-0.4 to 5.5), 7.7% sire calving ease (6.1 to 9.0%), 6.7% daughter calving ease (5.0 to 8.3%), 8.7% sire stillbirth (7.9 to 10.6%), 6.1% daughter stillbirth (4.9 to 8.2%), and 1.0% daughter pregnancy rate (-2.7 to 3.2%).

Cows. The experiment was conducted with lactating Holstein cows (primiparous and multiparous) housed at the University of Florida Dairy Unit (Hague). Embryo transfers were performed from June to October 2011. Cows were housed in freestall barns equipped with fans and sprinklers, fed a TMR, and milked 2 times daily. Cows were subjected to a presynchronization and Ovsynch-56 protocol beginning at 38 to 44 DIM as described for experiment 1. Cows diagnosed as nonpregnant were subjected to additional rounds of Ovsynch-56.

Cows were eligible for the experiment if they were scheduled for first service or had been either previously inseminated or received an embryo and were diagnosed nonpregnant. Each week, eligible cows were examined for the presence or absence of a CL by examination of the ovaries per rectum using an Aloka 500 ultrasound scanner equipped with a 5-MHz linear-array transducer (Aloka, Tokyo, Japan). If a CL was present, the cow was randomly assigned within pair of cows to treatment and received an embryo that had been cultured in BBH7 or SOF-BE1. Cows received an epidural block consisting of 5 mL of 2% (wt/vol) lidocaine, and a single embryo of the respective treatment was transferred to the uterine horn ipsilateral to the ovary with the CL via the transcervical method. Pregnancy was diagnosed by ultrasound at d 32 and by rectal palpation at d 46 and 76. Whether cows calved or experienced pregnancy loss after d 76 was recorded, as was calving difficulty (as for experiment 1) and occurrence of retained placenta, uterine prolapse, and metritis.

Statistics

Continuous data were analyzed by least-squares ANOVA using the GLM procedure of SAS (version 9.2., SAS Institute Inc., Cary, NC). Animal was considered random and other effects were considered fixed. Tests of homogeneity of variances between treatments

were performed by Levene's test and, if necessary (for birth weight in experiment 1), data were reanalyzed after performing log-transformation. Tests of significance were performed using the appropriate error terms as determined by calculation of expected mean squares. Differences between individual treatments of experiment 1 were determined by performing 2 orthogonal contrasts: AI versus ET and ET-F versus ET-V. Binomial data were analyzed by Pearson χ^2 analysis. Differences between treatments in survival of calves from birth to 150 d of age were determined using the LIFETEST procedure of SAS. For experiment 1, tests of homogeneity were based on comparison of AI to both ET groups and of ET-F to ET-V.

RESULTS

Experiment 1: Characteristics of Offspring and Dams in an IVP Program Compared with an AI Program

Pregnancy Outcomes. Results are summarized in Table 1. A greater proportion of cows receiving a fresh embryo were pregnant at 76 d of gestation compared with AI cows or cows receiving a vitrified embryo (AI vs. ET, nonsignificant; ET-F vs. ET-V; $P < 0.001$). Of cows pregnant at d 76, 8.3 to 10.9% were lost due to death or culling. Of the remaining cows, 4.5 to 7.3% aborted between d 76 and term; we observed no effect of treatment. When excluding cows that were culled or died, the calving rate paralleled results for pregnancy rate at d 76. The proportion of cows receiving a fresh embryo that calved was higher than for AI cows or cows receiving a vitrified embryo (AI vs. ET, nonsignificant; ET-F vs. ET-V, $P < 0.001$).

As shown in Table 1, gestation length was longer for cows receiving a vitrified embryo than for cows receiving a fresh embryo or AI (AI vs. ET, $P < 0.05$; ET-F vs. ET-V; $P < 0.001$). Differences between treatments were obscured somewhat by the practice of the farm to induce parturition for pregnancies where gestation length was 280 d or longer. Incidence of induced parturition was higher for ET than for AI ($P < 0.001$) and tended to be higher for cows receiving a vitrified embryo than for cows receiving a fresh embryo ($P = 0.06$). Among ET recipients, gestation length was longer ($P < 0.001$) for cows with induced parturition (282.0 ± 1.6 vs. 274.0 ± 0.7 d for ET-F, and 282.2 ± 1.2 vs. 277.2 ± 0.8 d for ET-V).

Postpartum Reproduction of Dams. Results are given in Table 2. The percentage of dams experiencing calving difficulty was higher for ET groups than AI ($P < 0.05$). We observed a tendency for incidence of retained placenta to be higher for ET than AI ($P = 0.06$) but treatment did not affect incidence of prolapse or

Table 1. Pregnancy outcomes as affected by embryo transfer (ET) with a fresh or vitrified embryo versus AI

Outcome	AI	ET-fresh	ET-vitrified	Probability	
				AI vs. ET	Fresh vs. vitrified
Pregnant at d 76 ¹	31.8 (48/151)	50.5 (48/95)	25.6 (46/180)	>0.10	<0.001
Culled or died before term ²	8.3 (4/48)	10.4 (5/48)	10.9 (5/46)	>0.10	>0.10
Aborted ³	4.5 (2/44)	4.7 (2/43)	7.3 (3/41)	>0.10	>0.10
Calving, of pregnant ³	95.5 (42/44)	95.3 (41/43)	92.7 (38/41)	>0.10	>0.10
Calving, of total except culls or died ⁴	28.6 (42/147)	45.6 (41/90)	21.7 (38/175)	>0.10	<0.001
Calving, of total ⁵	28.6 (42/151)	43.2 (41/95)	21.7 (38/180)	>0.10	<0.001
Gestation length (d) ⁶	274.9 ± 1.0	275.0 ± 1.0	279.5 ± 0.9	<0.05	<0.001
Induced parturition ⁷	0 (0/42)	14.6 (6/41)	31.6 (12/38)	<0.001	0.06

¹Percentage and fraction (in parentheses) of total cows receiving an embryo or inseminated.²Percentage and fraction (in parentheses) of pregnant cows at d 76.³Percentage and fraction (in parentheses) of pregnant cows at d 76 that were not culled or died.⁴Percentage and fraction (in parentheses) of total cows, after excluding those that were culled or died.⁵Percentage and fraction (in parentheses) of total cows.⁶Least squares means ± SEM.⁷Percentage and fraction (in parentheses) of cows that calved.

metritis. Induction of parturition increased frequency of calving difficulty [$P < 0.01$; 33.3% (6/18) vs. 7.8% (8/103)], retained placenta [$P < 0.001$; 22.2% (4/18) vs. 1.9% (2/103)], and metritis [$P < 0.01$; 50.0% (9/18) vs. 19.4% (20/103)] but not prolapse [5.6% (1/18) vs. 0% (0/103)]. As expected because of TAI, treatment had no effect on interval from calving to first service. In addition, we observed no effect of treatment on pregnancy rate at first service or services per conception.

Milk Production of Dams. As shown in Table 2, treatment had no effect on any measurement of milk production.

Characteristics of Calves. Results are shown in Table 3. The percentage of calves that were females was higher ($P < 0.001$) for ET than for AI cows. Treatment

had no effect on the percentage of cows giving birth to twins, which occurred for only one cow (bred by AI). We did, however, observe a reduced proportion of calves that were Holstein in the ET groups ($P < 0.05$), which reflects errors in identifying Holstein ovaries for harvesting of oocytes for in vitro fertilization.

The remaining analyses were performed on the subset of calves that were Holstein heifers (Table 3). Birth weight was not affected by treatment. However, the variance for BW tended ($P = 0.10$) to be affected by treatment, with greater variance in the ET groups. Differences between treatments in the distribution of birth weights are shown in Figure 1.

More calves were born dead, died, or were euthanized within the first 20 d of life for the ET groups than for

Table 2. Traits measured postpartum in cows giving birth as a result of embryo transfer (ET) or AI

Trait	AI	ET-fresh	ET-vitrified	Probability	
				AI vs. ET	Fresh vs. vitrified
Calving difficulty ¹	2.4 (1/42)	22.0 (9/41)	10.5 (4/38)	<0.05	>0.10
Prolapse ¹	0.0 (0/42)	2.4 (1/41)	0.0 (0/38)	>0.10	>0.10
Retained placenta ¹	4.8 (2/42)	22.0 (9/41)	10.5 (4/38)	0.06	>0.10
Metritis ¹	16.7 (7/42)	24.4 (10/41)	31.6 (12/38)	>0.10	>0.10
Interval to first service (d) ²	76.6 ± 0.5	76.5 ± 0.6	76.9 ± 0.6	>0.10	>0.10
Culled before breeding ¹	9.5 (4/42)	12.2 (5/41)	15.6 (6/38)	>0.10	>0.10
Pregnant at first service ³	26.3 (10/38)	16.7 (6/36)	21.9 (7/32)	>0.10	>0.10
Services per conception ²	2.5 ± 0.3	3.0 ± 0.4	2.9 ± 0.3	>0.10	>0.10
Milk yield ² (kg/d)	34.9 ± 1.0	35.5 ± 1.6	34.8 ± 1.1	>0.10	>0.10
Fat percentage ²	3.5 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	>0.10	>0.10
Protein percentage ²	2.9 ± 0.02	2.9 ± 0.04	3.0 ± 0.03	>0.10	>0.10
Lactose percentage ²	4.7 ± 0.02	4.7 ± 0.02	4.7 ± 0.02	>0.10	>0.10

¹Percentage and fraction (in parentheses) of total cows calving.²Least squares means ± SEM.³Percentage and fraction of cows that were inseminated.

Table 3. Traits of calves born as a result of embryo transfer (ET) or AI

Trait	AI	ET-fresh	ET-vitrified	Probability	
				AI vs. ET	Fresh vs. vitrified
Female calves ¹	51.2 (22/43)	92.7 (38/41)	92.1 (35/38)	<0.001	>0.10
Twin calves ²	2.4 (1/42)	0.0 (0/41)	0.0 (0/38)	>0.10	>0.10
Holstein calves ¹	100 (43/43)	82.9 (34/41)	92.1 (35/38)	<0.05	>0.10
Birth weight, Holstein heifers (kg) ³	41.7 ± 0.9	42.5 ± 1.2	45.3 ± 1.5	>0.10	>0.10
Holstein heifers, died <20 d of life ¹	4.5 (1/22)	19.4 (6/31)	25.0 (8/32)	0.06	>0.10
Holstein heifers, died >20 d of life to AI ¹	0.0 (0/21)	20.0 (5/25)	12.5 (3/24)	<0.05	>0.10
Holstein heifers, culled for poor growth, >20 d of life to AI ⁴	23.8 (5/21)	16.0% (4/25)	4.3 (1/23)	>0.10	>0.10
Holstein heifers, died or culled before AI ¹	27.3 (6/22)	48.4 (15/31)	37.5 (12/32)	>0.10	>0.10
Holstein heifers, age at first service, d ⁵	387.6 ± 3.7	385.9 ± 3.7	395.6 ± 3.3	>0.10	0.06
Holstein heifers, pregnant at first service ⁶	37.5 (6/16)	31.2 (5/16)	40.0 (8/20)	>0.10	>0.10
Holstein heifers, culled for reproduction ⁷	6.3 (1/16)	6.3 (1/16)	15.0 (3/20)	>0.10	>0.10
BW, 0–15 mo (kg) ⁵	96.3 ± 2.8	98.3 ± 3.8	94.7 ± 4.7	>0.10	>0.10
Body height, 0–15 mo (cm) ⁵	113 ± 1.0	112 ± 1.3	113 ± 0.8	>0.10	>0.10
BW/height, 0–15 mo (kg/cm) ⁵	0.86 ± 0.03	0.89 ± 0.4	0.86 ± 0.03	>0.10	>0.10
Age at first calving (d) ⁵	686 ± 159 (16)	663 ± 16 (16)	698 ± 14 (20)	>0.10	0.10
Daily milk yield, 0–120 DIM (kg) ^{5,8}	28.0 ± 1.8	25.3 ± 1.8	24.7 ± 1.8	>0.10	>0.10
Fat percentage ^{5,8}	3.5 ± 0.1	3.6 ± 0.1	3.7 ± 0.6	>0.10	>0.10
Protein percentage ^{5,8}	3.0 ± 0.03	3.0 ± 0.03	3.0 ± 0.03	>0.10	>0.10
Lactose percentage ^{5,8}	4.8 ± 0.04	4.7 ± 0.04	4.8 ± 0.04	>0.10	>0.10

¹Percentage and fraction (in parentheses) of total born.²Percentage and fraction (in parentheses) of cows calving.³Means ± SEM.⁴Percentage and fraction (in parentheses) of calves alive after 20 d.⁵Least squares means ± SEM.⁶Percentage and fraction (in parentheses) of total heifers inseminated.⁷Percentage and fraction (in parentheses) of cows that were inseminated.⁸n = 12 (AI), 12 (ET-fresh), and 15 (ET-vitrified).

AI ($P = 0.06$). Similarly, the proportion of calves that either died or were culled for poor health after 20 d of age was greater for the ET groups than for AI ($P < 0.05$). Survival curves for calves are shown in Figure 2A. Curves were heterogeneous between ET and AI ($P < 0.05$) but not different between ET-F and ET-V.

There was no effect of ET versus AI on age at first service or on the percentage of heifers pregnant at first service but age at first service tended to be higher ($P = 0.06$) for ET-V heifers than for ET-F heifers. Treatment did not affect the proportion of heifers culled for reproductive reasons. There were also no effects of treatment or treatment $\times$ age on BW, height, or the BW-to-height ratio. Treatments did not differ in milk yield or composition during the first 120 DIM of the first lactation (Table 3).

Given that parturition was induced in some ET cows but in none of the AI cows, data from Holstein heifers were scrutinized to determine whether differences between ET and AI for calf characteristics were the result of induction of parturition. In the absence of induced parturition, we observed no difference in birth weight between AI (41.7 ± 1.6 kg), ET-F (41.7 ± 1.1 kg), and ET-V (43.9 ± 1.3 kg). Among ET calves, birth weight

was greater ($P < 0.01$) for calves born following induced parturition (52.0 ± 2.9 kg vs. 42.8 ± 1.1 kg) but there was no interaction with treatment. All 6 of the ET-F calves and 5 of 8 of the ET-V calves that died in the first 20 d of age were from cows in which parturition was not induced. Calf losses in the first 20 d of age for calves from cows in which parturition was not induced were 4.5% for AI, 20.7% (6/29) for ET-F, and 22.7% (5/22) for ET-V (AI vs. ET; $P = 0.07$). Similarly, 4 of 5 ET-F calves and 2 of 3 ET-V calves that died or were culled for disease from >20 d of age to AI were from cows in which parturition was not induced. Calf losses to death or culling for disease from >20 d of age for calves until AI from cows in which parturition was not induced were 0.0% (0/21) for AI, 23.5% (4/17) for ET-fresh, and 18.2% (2/11) for ET-vitrified (AI vs. ET; $P < 0.05$).

Experiment 2: Outcome of ET as Modified by Culture Medium

Embryonic Development. Embryos were produced using X-sorted semen and cultured in either SOF-BE1 or BBH7 in 15 replicates (2,245 to 2,308 oocytes per

Figure 1. Distribution of birth weights for calves born as a result of AI, embryo transfer using a fresh embryo (ET-F), or embryo transfer using a vitrified embryo (ET-V). Points represent individual calves and the horizontal bars represent means. The variance of birth weights tended ($P = 0.10$) to be different between treatments.

medium). Culture medium had no effect on cleavage rate ($59.6 \pm 1.2\%$ for SOF-BE1 and $55.2\% \pm 1.2\%$ for BBH7) but we observed a tendency ($P = 0.105$) for the percentage of oocytes to develop to embryos at the blastocyst stage to be lower for SOF-BE1 ($9.8 \pm 0.8\%$) than for BBH7 ($11.9 \pm 0.8\%$).

Pregnancy Outcomes. Results are summarized in Table 4. Numerically, pregnancy rates and calving rates were greater for cows receiving an embryo produced in SOF-BE1 than an embryo produced in BBH7. This difference approached significance ($P = 0.08$) for pregnancy rate at 32 d of gestation but otherwise was not significant. We observed no effect of treatment on pregnancy loss between d 32 and 76 or between d 76 and term. More pregnancies were lost between d 32 and 76 of gestation (25%, 26 of 104 pregnancies) than between d 76 and term (5.8%, 4 of 69 cows; $P < 0.001$).

We detected no difference between cows receiving an SOF-BE1 or BBH7 embryo in gestation length, the percentage of cows in which parturition was induced, or the percentage of cows that experienced calving difficulty, retained placenta, prolapse, or metritis. Gestation length was longer ($P < 0.001$) for cows in which parturition was induced (281.4 ± 0.7 d) than for those not induced (275.4 ± 0.5 d). The incidence of

Figure 2. Survival curves for calves in experiments 1 (A) and 2 (B). For experiment 1, curves represent values for AI (solid line), embryo transfer with a fresh embryo (dashed line), and embryo transfer with a vitrified embryo (dotted line). Curves were heterogeneous between embryo transfer and AI ($P < 0.05$) but not different between fresh and vitrified. For experiment 2, curves represent values for calves produced as a result of embryo transfer using Block-Bonilla-Hansen 7 (BBH7) culture medium (dashed line) or embryo transfer using synthetic oviduct fluid-bovine embryo 1 (SOF-BE1) culture medium (solid line). Curves were not significantly different.

calving difficulty did not differ between those cows in which parturition was induced (18.2%, 4/22) compared with those not induced (16.3%, 7/43). Although not significant, the incidence of retained placenta, prolapse, or metritis tended to be higher for cows in which parturition was induced (31.8%, 7/22) than for those not induced (20.9%, 9/43).

Characteristics of Calves. The percentage of calves that were females was 89.2% (58/65) and was not affected by treatment. All calves were singletons and 93.8% (61/65) were Holsteins. Subsequent analyses were conducted with the subset of Holstein heifer calves ($n = 24$ BBH7 and 30 SOF-BE1 calves). Birth

Table 4. Pregnancy outcomes following transfer of embryos produced in either synthetic oviduct fluid–bovine embryo 1 (SOF-BE1) or Block-Bonilla-Hansen 7 (BBH7) culture medium

Outcome	SOF-BE1	BBH7	<i>P</i> -value
Pregnant at d 32, of total ¹	54.9 (56/102)	42.9 (48/112)	0.08
Pregnant at d 46, of total ¹	44.1 (45/102)	35.7 (40/112)	>0.10
Pregnant at d 76, of total ¹	42.2 (43/102)	31.3 (35/112)	>0.10
Pregnancy loss, d 32 to 76	23.2 (13/56)	27.0 (13/48)	>0.10
Calving, of total ²	36.7 (36/98)	27.1 (29/107)	>0.10
Pregnancy loss, d 76 to term ³	7.7 (3/39)	3.3 (1/30)	>0.10
Gestation length (d) ⁴	277.3 ± 1.0	277.6 ± 0.7	>0.10
Induced labor ⁵	38.9 (14/36)	27.6 (8/29)	>0.10
Calving difficulty ⁵	13.9 (5/36)	20.7 (6/29)	>0.10
Retained placenta, prolapse, or metritis ⁵	19.4 (7/36)	31.0 (9/29)	>0.10

¹Percentage and fraction (in parentheses) of total cows receiving an embryo.

²Percentage and fraction (in parentheses) of total cows receiving an embryo after excluding 9 cows removed from herd before calving.

³Percentage and fraction (in parentheses) of pregnant cows at d 76 after excluding 9 cows removed from herd before calving.

⁴Least squares means + SEM.

⁵Percentage and fraction (in parentheses) of total cows calving.

weight was higher ($P < 0.05$) for BBH7 calves than for SOF-BE1 calves, and was higher ($P < 0.001$) for calves from dams in which parturition was induced than from dams in which parturition was not induced (47.1 ± 1.1 vs. 42.0 ± 0.8 kg). We observed no significant effect of treatment on the percentage of calves that died or were euthanized <20 d of age [25% (6/24) for BBH7 and 13.3% (4/30) for SOF-BE1] or that died or were euthanized after 20 d of age [5.5% (1/18) for BBH7 vs. 3.8% (1/26) for SOF-BE1]. The survival curves for the 2 treatments did not deviate from homogeneity (Figure 2B). Although not significant, the incidence of calf death was lower for calves born following induced parturition (15.8%, 3/19) compared with those born without induced parturition (25.7%, 9/35).

DISCUSSION

To our knowledge, this is the first report of differences between calves born from AI and those born as a result of IVP-ET as adults, and is the first report to examine the consequences of ET for subsequent function of the dam. Conclusions are limited by the small sample size and the fact that different sires were used for AI and ET. Nonetheless, several conclusions can be made: calves born as a result of transfer of IVP embryos were more likely to be born dead or to die in early life than calves born as a result of AI; characteristics of the ET calf were affected by the culture system used for IVP; and there were few consequences to the dam of carrying a fetus derived by IVP-ET.

The most striking difference between ET and AI calves was the large incidence of death losses in the first 20 d of life. In the first experiment, for example, 22.2%

of ET Holstein heifer calves were born dead or died by 20 d of age. The incidence of death losses before 20 d of age in the AI Holstein heifer calves (4.5%) was much lower and was similar to the 3.6% value for incidence of stillbirths in a large data set of 10.8 million Holstein cows bred using conventional semen (Norman et al., 2010). The difference between ET and AI is not due to induction of parturition in cows having gestation lengths >280 d because the incidence of calf death was higher for pregnancies in which parturition was not induced. Perhaps abnormalities in fetal development resulted in a shorter gestation length. The difference between ET and AI was also not due to the use of sexed semen in the former group because the incidence of stillbirths in calves born from cows inseminated using sexed semen was lower than for cows inseminated with conventional semen (Norman et al., 2010). We cannot rule out sire differences between the ET and AI groups but the high incidence of early death losses was also apparent in the second experiment (18.5%) even though sires used for IVP were different in the first experiment. Moreover, differences in PTA for calving ease and stillbirths between ET and AI groups were small.

In an earlier experiment from our laboratory and on the same farm (Block et al., 2003), 18% of calves born following ET using IVP embryos were born dead or died within 24 of birth. Others have also reported increased incidence of stillbirths and neonatal mortality, ranging from 7.8 to 14.4%, for IVP calves (Kruip and Den Haas, 1997; van Wagtenonk-de Leeuw et al., 1998; Numabe et al., 2000).

Few differences in survival, growth, or subsequent milk yield were observed between ET and AI calves among those surviving to >20 d of age although the

increased death loss for ET calves persisted in the period from >20 d to AI. Research with a larger numbers of animals will provide a better indication of whether developmental programming caused by culture in vitro during the first 7 d of development has effects that persist beyond the neonatal period.

Another problem encountered with IVP-ET was the birth of a small number of crossbred animals because of misidentification of ovaries at collection. Two other problems associated with IVP—skewed sex ratio (van Wagtendonk-de Leeuw et al., 1998; Block et al., 2003) and birth of calves with large birth weights (Behboodi et al., 1994; Kruip and Den Haas, 1997; van Wagtendonk-de Leeuw et al., 1998; Numabe et al., 2000; Breukelman et al., 2005)—were generally avoided. Use of sexed semen resulted in 89.8% of calves produced by ET being female. This value is very similar to the value of 89.3% female for cows bred by sexed semen using AI (Norman et al., 2010). Large birth weights were not a frequent occurrence in the current study, perhaps because parturition was induced at 280 d of gestation. Nonetheless, we observed greater variation in birth weights among ET calves than among AI calves, with one ET calf being exceptionally large (72 kg) and some calves being born at smaller weights than AI calves.

There are indications that the characteristics of calves born as a result of IVP-ET depend upon the culture system. In particular, birth weights were higher for calves born as a result of embryo culture in BBH7 than for calves born as a result of culture in SOF-BE1. We also observed a nonsignificant tendency for calf death losses to be higher for BBH7 calves (Figure 2B). One implication of this finding is that the likelihood of calf abnormalities following IVP-ET will depend upon the culture system. Identification of specific molecules regulating early embryonic development may lead to improvements in development of embryos in vitro to reduce some of the problems associated with IVP-ET in the current study. Among candidate molecules are IGF1 and colony stimulating factor 2, both of which have been reported to increase initial pregnancy rate after transfer into recipients and reduce pregnancy loss later in gestation (Block et al., 2003; Block and Hansen, 2007; Loureiro et al., 2009).

One difference between embryos produced in vitro and those produced by AI was that the former were derived from oocytes harvested from follicles at a variety of different stages of development and atresis, whereas the latter were derived from ovulated oocytes. It is possible that the incidence of calf death loss following ET might have been different if a different source of oocytes was used for IVP. The importance of oocyte source on characteristics of the resulting calf has not been studied in depth. In one recent study, the transcriptome of IVP

blastocysts was very similar between embryos produced using oocytes recovered postmortem and embryos produced using oocytes harvested from FSH-treated cows using transvaginal, ultrasound-guided aspiration (Plourde et al., 2012).

CONCLUSIONS

Although calves born as a result of IVP-ET are more likely to experience death in early life, we found few consequences to the dam of carrying a fetus derived by IVP-ET. Indeed, the incidence of postpartum diseases and subsequent milk yield were not different between cows carrying fetuses derived from ET or AI. Thus, use of IVP-ET for improvement of fertility or genetic selection can be performed without a major compromise of function of the recipient cow. Nonetheless, as pointed out here, the value of using IVP-ET to enhance fertility or genetic selection will be reduced if the system used to produce an embryo for transfer results in the type of calf death losses reported here. Further optimization of culture systems to produce embryos in vitro could improve the benefits of embryo technology. As noted here, it is not sufficient to assess embryo production systems simply by measuring blastocyst production rates or even initial pregnancy rates following transfer. Rather, accurate assessment of the benefits of proposed improvements to IVP will necessitate carrying pregnancies to term and assessing the health of the offspring.

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