Efficacy of in vitro embryo transfer in lactating dairy cows using fresh or vitrified embryos produced in a novel embryo culture medium¹

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ABSTRACT

Objectives were to determine whether pregnancy success could be improved in lactating cows with timed embryo transfer when embryos were produced in vitro using a medium designed to enhance embryo development and survival after cryopreservation. In experiment 1, embryos ($n = 569$ to 922) were cultured in either modified synthetic oviduct fluid or a serum-free medium, Block-Bonilla-Hansen-7 (BBH7). Development to the blastocyst stage was recorded at d 7, and selected blastocysts ($n = 79$ to 114) were vitrified using open pulled straws. Culture of embryos in BBH7 increased development to the blastocyst stage (41.9 ± 2.0 vs. $14.7 \pm 2.0\%$) and advanced blastocyst stages (expanded, hatching, hatched; 31.1 ± 1.3 vs. $6.4 \pm 1.3\%$) at d 7 and resulted in higher hatching rates at 24 h postwarming compared with embryos cultured in modified synthetic oviduct fluid (59.0 ± 0.5 vs. $26.7 \pm 0.5\%$). In experiment 2, embryos were produced using X-sorted semen and cultured in BBH7. At d 7 after insemination, embryos were transferred fresh or following vitrification. Lactating Holstein cows were either subjected to timed artificial insemination (TAI) on the day of presumptive ovulation or used as embryo recipients 7 d later. Embryo recipients received an embryo if a corpus luteum was present. The percentage of cows pregnant at d 32, 46, and 76 of gestation was higher among cows that received fresh embryos compared with TAI cows or cows that received vitrified embryos. At d 76, for example, the proportion and percentage pregnant was 47/150 (31.3%) for cows subjected to TAI, 48/95 (50.5%) for cows receiving fresh embryos, and 39/141 (27.7%) for cows receiving a vitrified embryo. No difference was observed in the percentage of cows pregnant among TAI cows and those that received vitri-

fied embryos. There was a service or transfer number $\times$ treatment interaction because differences in pregnancy rate between embryo transfer recipients and cows bred by TAI were greater for cows with more than 3 services or transfers. Pregnancy success in lactating cows can be improved by transferring fresh embryos produced in BBH7 compared with TAI. Moreover, no decline in fertility was observed when cryopreserved embryos were transferred compared with TAI. Embryo transfer is particularly efficacious for infertile cows that have previously experienced several failed breeding attempts.

Key words: cryopreservation, embryo transfer, fertility

INTRODUCTION

Embryo transfer can be an important tool for genetic improvement and fertility enhancement in dairy cattle and other animals (Hansen and Block, 2004). Its use as an assisted-reproduction technology is based on embryo transfer bypassing pregnancy failure caused by defects in ovulation, fertilization, embryonic development, and AI technique. Improvements in pregnancy rate using embryo transfer were demonstrated for lactating cows subjected to heat stress (Al-Katanani et al., 2002; Block et al., 2003; Rodrigues et al., 2004). In each of these studies, fertility in inseminated cows was low. When heat stress is not a contributing factor to pregnancy failure, pregnancy rates between embryo-transfer recipients and inseminated cows do not differ (Rodrigues et al., 2004; Sartori et al., 2006). In another study carried out throughout the year, pregnancy rates after embryo transfer were higher than for AI, and high body temperature caused a reduction in pregnancy rate in both groups (Demetrio et al., 2007).

The failure of embryo transfer to consistently improve fertility in lactating cows when fertility in inseminated cows is not very low could reflect reduced competence of the embryo used for transfer to establish and maintain pregnancy. This problem is exacerbated when the embryo is derived in vitro because an embryo produced by that process has altered molecular and cellular properties that reduce its capacity for survival.

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¹Conflict of interest: All authors have an ownership position in Cooley Biotech, LLC, Gainesville, Florida, the licensee of BBH7 medium.

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³BBH7 will be made available to researchers wishing to replicate the results herein.

ing cryopreservation and establishing pregnancy after transfer into recipients. The period of embryo culture is an important one for producing embryos with high competence for survival because transfer of in vitro-produced embryos into the sheep oviduct has been shown to enhance development, gene expression, and cryosurvival (Rizos et al., 2008), and the addition of specific molecules, such as colony-stimulating factor 2 and IGF-1 improved posttransfer survival of in vitro-produced embryos (Block et al., 2007; Loureiro et al., 2009).

Block-Bonilla-Hansen 7 (**BBH7**) is a defined, serum-free culture medium that was developed to increase the yield and cryotolerance of blastocysts produced in vitro. For the present experiment, it was hypothesized that BBH7 would increase embryo competence for development and cryosurvival, and that the resultant embryos would be of sufficient quality that transfer of either a fresh or vitrified embryo would increase pregnancy success of lactating cows. Furthermore, it was hypothesized that benefits of embryo transfer would be greater for cows that had been bred more than 3 times previously (i.e., repeat breeder cows) because defects in a subpopulation of these cows associated with failure of ovulation, fertilization, and early development would be bypassed.

MATERIALS AND METHODS

Materials

All materials were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ) unless specified otherwise. HEPES-Tyrode's lactate and in vitro fertilization (**IVF**)-Tyrode's lactate were purchased from Caisson Laboratories Inc. (Logan, UT) or Millipore (Billerica, MA). These media were used to prepare HEPES-Tyrode's albumin lactate pyruvate (**TALP**) and IVF-TALP, as described previously (Parrish et al., 1986). Oocyte collection medium consisted of tissue culture medium-199 with Hank's salts without phenol red and supplemented with 2% (vol/vol) bovine steer serum (Pel-Freez, Rogers, AR), 2 U/mL of heparin, 100 U/mL of penicillin-G, 0.1 mg/mL of streptomycin, and 1 mM glutamine. Oocyte maturation medium was TC-199 with Earle's salts (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) bovine steer serum, 2 µg of estradiol 17-β/mL, 20 µg of bovine FSH/mL (Folltropin-V; Bioniche, Belleville, ON, Canada), 22 µg of sodium pyruvate/mL, 50 µg of gentamicin sulfate/mL, and 1 mM glutamine. Percoll was from GE Healthcare (Chalfont St. Giles, UK). Modified synthetic oviductal fluid was purchased from Millipore (Billerica, MA). The formula was as described

by Takahashi and First (1992) except that phenol red and BSA were omitted. The synthetic oviductal fluid was modified (**mSOF**) before use to contain 1.0 mM alanyl-glutamine, 5.3 mM sodium lactate, 0.5 mM tri-sodium citrate, 2.77 mM myo-inositol, 0.5 mM fructose, 20 µL of essential AA/mL (Eagle's basal medium), and 10 µL of nonessential AA/mL (minimum essential medium). The medium BBH7 is a proprietary, serum-free culture medium developed by the University of Florida and licensed to Cooley Biotech LLC (Gainesville, FL). Gonadotropin-releasing hormone was Cystorelin from Merial (Duluth, GA), and PGF_{2α} was Lutalyse from Pfizer (New York, NY). Lidocaine was obtained from Pro Labs (St. Joseph, MO). Open pulled straws were from Mintube (Verona, WI). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA).

Experiment 1: Effect of Culture Medium on Blastocyst Development, Survival Following Vitrification, Cell Number and Differentiation, and Lipid Content

In Vitro Embryo Production. All procedures related to in vitro embryo production were as described previously (Soto et al., 2003) unless otherwise noted. Immature cumulus oocyte complexes (**COC**) were collected from abattoir-derived ovaries (Central Packing, Center Hill, FL). Harvested COC were matured for 21 to 24 h in a humidified atmosphere of 5% (vol/vol) CO₂ in air. Following maturation, COC were washed once in HEPES-TALP and then coincubated with a pool of sperm from 3 different bulls. To eliminate bull effects, a different combination of 3 bulls was used for each replicate. Spermatozoa and COC were coincubated for 8 h. Following fertilization, presumptive zygotes were cultured in groups of approximately 30 in 50-µL microdrops of either mSOF or BBH7. Culture drops were overlaid with mineral oil and placed at 38.5 °C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% (vol/vol) N₂ for 7 d. The proportion of oocytes that cleaved was recorded on d 3 after insemination. The proportion of oocytes that developed to the blastocyst stage (i.e., all blastocysts including those that were nonexpanded, expanded, hatching, and hatched) and advanced blastocyst stage (expanded, hatching, and hatched blastocysts) were recorded on d 7 after insemination. Embryos were produced in 12 replicates and used as described below.

Vitrification and Postwarming Survival. For 8 replicates, selected embryos were vitrified and warmed using the open pulled straw method as previously described (Vajta et al., 1998) with minor modifications. All steps were carried out with media kept on a warmed

stage set at 39°C. Blastocyst and expanded-blastocyst stage embryos were harvested from culture at d 7 after insemination and washed twice in holding medium (tissue culture medium-199 with Hanks' salts supplemented with 10% vol/vol fetal bovine serum). Embryos were then incubated in holding medium containing 7.5% (vol/vol) ethylene glycol and 7.5% (vol/vol) dimethyl sulfoxide for 3 min. Following incubation, embryos were moved to a solution of holding medium containing 16.5% (vol/vol) ethylene glycol and 16.5% (vol/vol) dimethyl sulfoxide and loaded into open pulled straws. After 25 s, the open pulled straws were plunged directly into liquid nitrogen. For warming, open pulled straws were immersed into holding medium plus 0.3 M sucrose and the embryos were expelled from the straws. Embryos were immediately moved to another solution of holding medium plus 0.3 M sucrose and held for 5 min. Embryos were subsequently washed in holding medium plus 0.2 M sucrose and holding medium alone for 5 min each. Embryos were cultured in 25- μ L microdrops of either mSOF or BBH7 supplemented with 10% fetal bovine serum and 50 μ M dithiothreitol overlaid with mineral oil for 72 h in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 38.5°C. Re-expansion and hatching rates (including both hatching and hatched embryos) were recorded at 24, 48, and 72 h postwarming.

Determination of Cell Allocation and Lipid Content. For 3 replicates, embryos were used to determine either cell allocation to the inner cell mass and trophectoderm or lipid content. At d 7 after insemination, blastocysts and expanded blastocysts were harvested from culture and randomly divided into 2 groups with similar numbers of embryos of each stage represented in each group. Differential staining was used to determine allocation of cells to the inner cell mass and trophectoderm. Embryos used for differential staining were washed 3 times in 50- μ L drops of 10 mM KPO₄, pH 7.4, containing 0.9% (wt/vol) NaCl (PBS) and 1 mg/mL of polyvinylpyrrolidone (PVP) (Eastman Kodak, Rochester, NY). To label trophectoderm cells (TE), embryos were placed into 500 μ L of PBS-PVP containing 0.5% (vol/vol) Triton X-100 and 100 μ g/mL of propidium iodide for 30 s at 39°C. Embryos were washed immediately through 3 wells of a 4-well plate each containing 500 μ L of PBS-PVP. To fix embryos and stain inner cell mass cells (ICM), embryos were then incubated in a 50- μ L drop of PBS-PVP containing 4% (wt/vol) paraformaldehyde and 10 μ g of Hoechst 33258/mL for 15 min at room temperature. Embryos were washed 3 times in PBS-PVP, mounted on 10% (wt/vol) poly-L-lysine coated slides and covered with coverslips. Labeling of propidium iodide and Hoechst 33258 nuclei was observed with a Zeiss Axioplan 2 epi-

fluorescence microscope (Zeiss, Göttingen, Germany). Each embryo was analyzed for the number of ICM (blue nuclei), number of TE cells (pink nuclei), and total cell number (blue and pink nuclei) with a 20 $\times$ objective. Digital images were acquired with Zeiss AxioVision software and a high-resolution black and white Zeiss AxioCam MRm digital camera set to a standard exposure length. Cell counting was performed using ImageJ (<http://rsbweb.nih.gov/ij/>).

The degree of lipid was determined using Nile red (Invitrogen) as described previously (Leroy et al., 2005). All steps were performed in the wells of a 96-well plate. Embryos were washed 3 times in wells containing 100 μ L of PBS-PVP for 2 min each. Embryos were then transferred to 1 μ g/mL of Nile red dissolved in 0.1% (vol/vol) dimethyl sulfoxide in PBS-PVP. After 30 min, embryos were washed 3 times in PBS-PVP, mounted on slides with coverslips using ProLong Gold Antifade reagent (Invitrogen), and examined for fluorescence with a Zeiss Axioplan 2 epifluorescence microscope with Zeiss filter set 03 (fluorescein). As a negative control, some embryos were incubated in 100 μ L of absolute ethanol for 30 min to dissolve lipid before staining with Nile red. Digital images were acquired using Zeiss AxioVision software and a high-resolution black and white Zeiss AxioCam MRm digital camera set to a standard exposure length. Intensity of fluorescence per unit area was quantified using ImageJ (<http://rsbweb.nih.gov/ij/>).

Experiment 2: Pregnancy Rates Obtained Following Transfer of Either Fresh or Vitrified Embryos Produced by Culture in BBH7 Versus AI

In Vitro Embryo Production. Embryos were produced in vitro as described for experiment 1 with a few modifications as described below. Holstein COC were collected from abattoir-derived ovaries. Following maturation, COC were inseminated with X-sorted semen from 1 of 2 different Holstein bulls (ABS Global, Deforest, WI). Prior to insemination, COC were washed once in HEPES-TALP and then placed in groups of 30 into 50- μ L microdrops of IVF-TALP that contained 2 mM caffeine. For sperm preparation, semen was layered on top of a mini-Percoll gradient (300 μ L of 45% Percoll over 300 μ L of 90% Percoll) and then centrifuged at 600 $\times g$ for 10 min. The sperm pellet was placed into a 2-mL microcentrifuge tube containing 1 mL of HEPES-TALP modified to contain 10 mM caffeine and centrifuged for 5 min at 300 $\times g$. Following centrifugation, the supernatant was removed and the sperm pellet was diluted in IVF-TALP containing 2 mM caffeine. After sperm dilution, 20 μ L of the sperm solution and 3 μ L of 0.5

mM penicillamine, 0.25 mM hypotaurine, and 25 μ M epinephrine in 0.9% (wt/vol) NaCl were added to each fertilization drop. The final sperm concentration was 1×10^6 sperm/mL. Spermatozoa and COC coincubated for 8 h. Following fertilization, presumptive zygotes were cultured in 50- μ L microdrops of BBH7 overlaid with mineral oil in groups of 25 to 30 in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 38.5°C for 7 d.

For fresh embryos, grade-1 expanded blastocysts (Robertson and Nelson, 1998) were harvested at d 7 after insemination and loaded into 0.25-mL straws in transfer medium (HEPES-TALP containing 10% fetal bovine serum and 50 μ M dithiothreitol). Straws were placed into a portable incubator (Biotherm INC-12v, Cryologic, Victoria, Australia) set at 38.5°C and transported to the farm for transfer to recipients. For vitrified embryos, grade-1 expanded blastocysts were harvested at d 7 after insemination and vitrified using the open pulled straw method as described in experiment 1. At the farm, vitrified embryos were warmed as in experiment 1 and loaded into 0.25-mL straws in transfer medium. Straws containing vitrified embryos were placed into a portable incubator set at 38.5°C and held until transfer.

Animals. The experiment was conducted between May 8 and October 16, 2009, at the University of Florida Dairy Unit (Hague, FL; 29.77904 N, 82.48001 W). Primiparous and multiparous lactating Holstein cows were enrolled. Cows were housed in freestall barns equipped with fans and sprinklers. They were fed a TMR and milked 2 times daily. The experimental protocol received Institutional Animal Care and Use Committee approval before the experiment was initiated.

Eligible cows (included those that were first-service cows and those that were previously inseminated or received an embryo and were diagnosed nonpregnant) were enrolled into the experiment on a weekly basis (7 to 45/wk) during the 24-wk period and randomly assigned to 1 of 3 treatment groups: timed artificial insemination (**TAI**), timed-embryo transfer with a fresh embryo, or timed embryo transfer with a vitrified embryo. Cows were subjected to the Ovsynch-56 timed ovulation protocol (Brusveen et al., 2008). Specifically, cows received 100 μ g of GnRH, i.m., on d -10; 25 mg of PGF_{2 α} , i.m., on d -3; and 100 μ g of GnRH, i.m., at 56 h after PGF_{2 α} . For first-service cows only, the timed ovulation protocol was preceded by a presynchronization protocol (2 injections of 25 mg of PGF_{2 α} , i.m., 14 d apart), with the last injection 12 d before initiation of the Ovsynch-56 protocol. Moreover, first-service cows were palpated on the day of Ovsynch-56 initia-

tion. If they did not have any palpable structures on the ovaries, the Ovsynch-56 protocol was not initiated. Cows were palpated weekly and Ovsynch-56 started when the palpation diagnoses indicated the cow was experiencing estrous cycles.

Cows assigned to the TAI group were inseminated on d 0, approximately 16 h after the second GnRH injection of the Ovsynch-56 protocol, using unsorted semen from Holstein bulls selected by the farm. On d 7 after presumptive ovulation, the presence or absence of a corpus luteum (**CL**) was diagnosed for all cows enrolled in the experiment with an Aloka 500 ultrasound scanner equipped with a 5-MHz linear-array transducer (Aloka, Tokyo, Japan). Cows in the TAI group that did not have a CL on d 7 were removed from the experiment. All cows in the timed fresh and vitrified embryo transfer groups that were diagnosed as having a CL received an epidural block (5 mL of 2% lidocaine, wt/vol), 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. A total of 197 primiparous and 192 multiparous cows were used. The average DIM at the predicted date of ovulation was 153 d (range 74 to 593) and the average number of times cows were bred (including the experimental service or embryo transfer) was 2.5 (range 1 to 11).

Statistical Analysis

For experiment 1, data were analyzed by least-squares ANOVA using the GLM procedure of SAS (SAS for Windows, version 9.0, SAS Institute Inc., Cary, NC). The model included the main effects of replicate and treatment. Percentage data were subjected to arcsin transformation before analysis. All values reported are least squares means $\pm$ SEM. Probability values for percentage data are based on the analysis of arcsin-transformed data and least squares means are from analysis of untransformed data.

For experiment 2, data for the percentage of cows that became pregnant at d 32, 46, and 76, and the percentage of cows that lost their pregnancy between d 32 and d 76, were analyzed by logistic regression using the LOGISTIC procedure of SAS. The model included replicate, treatment, parity (1 vs. others), times bred (1 to 3 vs. >3; including the experimental insemination or transfer), treatment $\times$ parity, and treatment $\times$ times bred interactions. Data are presented as the actual proportions. Contrasts were used to analyze differences among the 3 treatment groups.

Table 1. Effect of culture medium on cleavage rate at d 3 and blastocyst development at d 7 after insemination¹

Treatment	Oocytes, n	Cleaved, %	Blastocysts, % of oocytes	Advanced blastocysts, % of oocytes ²
Block-Bonilla-Hansen 7	569	80.1 ± 1.4 ^a	41.9 ± 2.0 ^a	31.1 ± 1.3 ^a
Modified synthetic oviduct fluid	922	80.0 ± 1.4 ^a	14.7 ± 2.0 ^b	6.4 ± 1.3 ^b

^{a,b}Means with different superscripts in a column differ ($P < 0.001$).

¹Data represent LSM ± SEM.

²Advanced blastocysts were expanded, hatching, or hatched.

RESULTS

Experiment 1: Effect of Culture Medium on Blastocyst Development, Survival Following Vitrification, Cell Number and Differentiation, and Lipid Content

Culture medium had no effect on cleavage rate at d 3 after insemination. A greater ($P < 0.001$) proportion of oocytes cultured in BBH7 developed to the blastocyst and advanced blastocyst stages at d 7 after insemination than for oocytes cultured in mSOF (Table 1).

Culture medium did not affect re-expansion rates of vitrified embryos at any of the time points analyzed nor did culture medium affect hatching rates at 48 and 72 h postwarming. The hatching rate of vitrified embryos cultured in BBH7 was higher ($P < 0.001$) at 24 h postwarming than for embryos cultured in mSOF (Table 2).

As depicted in Table 3, cell proliferation and cell allocation of blastocysts harvested at d 7 after insemination was affected by culture medium. Specifically, blastocysts that were produced in BBH7 had increased ($P < 0.01$) total cell number compared with embryos cultured in mSOF. Much of the increase in total cell number was the result of an increase ($P < 0.001$) in number of cells in the ICM. This was reflected in the TE:ICM ratio, which was reduced ($P < 0.05$) for embryos cultured in BBH7 compared with those cultured in mSOF. There was a tendency ($P = 0.10$) for embryos cultured in BBH7 to have more cells in the TE than for embryos cultured in mSOF. There was no difference in

lipid content of blastocysts produced in BBH7 versus those cultured in mSOF (Table 3).

Experiment 2: Pregnancy Rates Obtained Following Transfer of Either Fresh or Vitrified Embryos Produced by Culture in BBH7 Versus AI

A main effect of treatment ($P < 0.01$) was found on the percentage of cows that became pregnant at d 32, 46, and 76 of gestation (Table 4). In particular, the percentage of cows that were pregnant was higher for those receiving a fresh embryo than for those that were either artificially inseminated or received a vitrified embryo (Table 4). The percentage of cows that became pregnant was not different between cows that were either AI or received vitrified embryos.

Cows were categorized according to whether the number of times a cow was inseminated or received an embryo (including the experimental insemination or transfer) was 1 to 3 or >3 . This categorization was used because cows that are bred at least 3 times without becoming pregnant are often considered repeat-breeder cows. A times bred $\times$ treatment interaction was observed for the proportion of cows pregnant on d 32 ($P < 0.01$), 46 ($P < 0.05$), and 76 ($P = 0.07$). As represented in Figure 1, this interaction reflects that cows bred >3 times have a lower rate of pregnancy success than do cows bred ≤ 3 times if the cows are inseminated, but not if the cows receive a fresh or vitrified embryo.

An effect of parity ($P < 0.01$) was also observed, with the percentage of cows pregnant being higher for primiparous cows at d 32 (48.7 vs. 31.8%), 46 (45.4 vs.

Table 2. Effect of culture medium on reexpansion and hatching following vitrification at 24 to 72 h postthaw¹

Treatment	Blastocysts, n	Reexpansion, %			Hatching, %		
		24 h	48 h	72 h	24 h	48 h	72 h
Block-Bonilla-Hansen 7	114	91.9 ± 1.4 ^a	91.4 ± 0.4 ^a	91.4 ± 0.7 ^a	59.0 ± 0.5 ^a	79.7 ± 0.6 ^a	83.6 ± 0.7 ^a
Modified synthetic oviduct fluid	79	91.2 ± 1.4 ^a	83.5 ± 0.4 ^a	81.8 ± 0.7 ^a	26.7 ± 0.5 ^b	71.2 ± 0.6 ^a	76.8 ± 0.7 ^a

^{a,b}Means with different superscripts within a column differ ($P < 0.001$).

¹Data represent least squares means ± SEM.

Table 3. Effect of culture medium on blastocyst lipid content, cell number, and cell allocation at d 7 after insemination¹

Item	BBH7	mSOF ²
Lipid content, U	886 ± 14	867 ± 14
Total cell number	166 ± 10.7	119 ± 12.1**
ICM cells, n	59 ± 3	32 ± 4***
TE cells, n	107 ± 9	86 ± 10†
TE:ICM	2.1 ± 0.2	2.8 ± 0.3*

¹BBH7 = Block-Bonilla-Hansen 7; mSOF = modified synthetic oviduct fluid; ICM = inner cell mass; and TE = trophectoderm. Data represent least squares means ± SEM for 37 to 38 embryos per group in 3 replicates.

²Treatment effects: † $P = 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

29.2%), and 76 (42.6 vs. 26.7%). There was no interaction between parity and treatment. Cows were categorized as to whether they were in the hot season (May to September; $n = 327$) or cool season (October; $n = 62$). Season (41.0% in hot season vs. 37.1% in cool season for percent pregnant at d 32) and season × treatment interaction did not affect percentage pregnant.

Treatment did not affect pregnancy loss between d 32 and 76 of gestation. Pregnancy losses for cows that were artificially inseminated, received fresh embryos, or received vitrified embryos were 8/55 (14.5%), 5/53 (9.4%), and 7/46 (15.2%), respectively.

DISCUSSION

To develop a practical system for using embryo transfer to improve fertility, the costs of embryo production must be kept as low as possible. In this regard, the embryo produced in vitro is preferable to embryos produced by superovulation because the former can be produced inexpensively using oocytes recovered from ovaries obtained from an abattoir. Compared with its counterpart produced in vivo, the bovine embryo produced in vitro suffers from increased susceptibility to cryological damage and reduced pregnancy rates after transfer into recipients, even in the absence of cryopreservation (Rizos et al., 2008; Hansen et al., 2010). The BBH7 culture medium was developed to produce bovine embryos in vitro that were capable of surviving cryopreservation. Results presented indicate that blastocysts produced in BBH7 have superior resistance to cryological damage when compared with blastocysts cultured in another popular medium, mSOF, as determined by a reduction in the proportion of embryos that experienced delayed hatching after thawing. Furthermore, an increased number of blastocysts was observed for embryos cultured in BBH7 compared with embryos cultured in mSOF. The competence of fresh blastocysts produced in BBH7 to establish and maintain pregnancy after transfer into lactating cows was high enough to

Figure 1. Interaction between number of times bred and treatment on pregnancy success at d 32, 46, and 76 of gestation. Cows were categorized as to whether the experimental service or transfer was the first, second or third since calving (1 to 3) or greater than the third (>3). Cows were bred by AI (open bars), embryo transfer using a fresh embryo (hatched bar), or embryo transfer using a vitrified embryo (black bar). The number of cows per treatment varied from 70 to 118 for cows bred 1 to 3 times and 25 to 33 for cows bred >3 times.

Table 4. Proportion of cows pregnant following transfer of either fresh or vitrified embryos produced in vitro compared with timed AI at d 32, 46, and 76 of gestation

Treatment	Proportion of cows pregnant, n/total (%)		
	d 32	d 46	d 76
Timed AI	56/151 (37.1) ^a	53/151 (35.1) ^a	47/150 (31.3) ^a
Timed embryo transfer, fresh	54/96 (56.3) ^b	50/95 (52.6) ^b	48/95 (50.5) ^b
Timed embryo transfer, vitrified	47/142 (33.1) ^a	42/142 (29.6) ^a	39/141 (27.7) ^a

^{a,b}Means with different superscripts within a column differ ($P < 0.01$).

allow an improvement of pregnancy rates over those achieved with TAI. Results highlight the utility of using embryo transfer as a reproductive management technique to improve fertility in lactating dairy cows.

To date, experiments in which pregnancy rates were higher for lactating embryo transfer recipients than for cows bred by AI involved heat stress for all (Al-Katanani et al., 2002; Block et al., 2003; Rodriques et al., 2004) or a fraction of the cows (Demetrio et al., 2007). When heat stress was not a factor, no differences in pregnancy rate were found between embryo transfer recipients and inseminated cows (Sartori et al., 2006). The present experiment, which demonstrated an improvement in pregnancy rate caused by transfer of fresh embryos (experiment 2), was performed in Florida during a period of the year (May to October) in which heat stress is prevalent. It is not possible to know for certain, but we interpret the improvement in pregnancy rate as a beneficial effect of embryo transfer independent of heat stress effects on the recipient. This assertion is made because there appeared to be little effect of heat stress on fertility. Season did not have a significant effect on fertility in experiment 2.

It is not known why, in contrast to earlier studies (Rodriques et al., 2004; Sartori et al., 2006), pregnancy rates for cows receiving fresh embryos were higher than for cows subjected to TAI in the cool season. The increase in the number of ICM cells and the increase in the TE:ICM ratio in blastocysts produced in BBH7 compared with those produced in mSOF may have contributed to the high pregnancy rate achieved in cows receiving fresh embryos. About 25% of in vitro-produced embryos fail to develop an embryonic disc by d 14 (Fischer-Brown et al., 2004; Block et al., 2007), and it is possible that increases in the number of ICM cells increased the proportion of embryos that developed an embryonic disc. Increases in cell number may have contributed to the cryosurvival of embryos in this study. Although lipid accumulation was implicated as a cause for reduced cryosurvival of in vitro-produced bovine embryos (Pereira and Marques, 2008), there was no evidence that the increased survivability of embryos produced in BBH7 was due to reduced lipid accumulation.

In previous studies, pregnancy rates for lactating cows that received a cryopreserved, in vitro-produced embryo varied from 4.8% (produced in CR1aa medium; Ambrose et al., 1999), 6.5% (modified KSOM medium; Al-Katanani et al., 2002), and 18.8% (medium not stated; Drost et al., 1999). In the present experiments, 27.7% of cows that received a vitrified embryo were pregnant at d 76 of gestation. Thus, acceptable pregnancy rates can be achieved with transfer of vitrified embryos produced in vitro. Nevertheless, pregnancy rates for cows receiving a vitrified embryo were not higher than for TAI. This result implies that whereas cryopreserved, in vitro produced embryos can be successfully used as a means of genetic improvement, survival of such embryos is not yet high enough to make the transfer of cryopreserved embryos in lactating cows a useful procedure for increasing pregnancy rates compared with TAI, at least when fertility is high.

For experiment 2, pregnancy rate for inseminated cows was lower for those that had been bred previously >3 times. Such a result is consistent with other findings that repeat-breeder cows have lower fertility than do cows bred at lower service numbers (García-Ispuerto et al., 2007; Yusuf et al., 2010). Several factors have been related to low fertility in repeat-breeder cows, including uterine infectious disease, cystic ovaries, reduced progesterone concentrations, and increased free radical production (Moss et al., 2002; Rizzo et al., 2007; Kendall et al., 2009). Transfer of a fresh or vitrified embryo overcame the inherent infertility in repeat-breeder cows in experiment 2. In particular, pregnancy rate was not lower for embryo transfer recipients that had been bred >3 times previously than for cows bred less often. Thus, differences in pregnancy rates among cows receiving embryos and those subjected to TAI were greater for repeat-breeder cows than for cows bred 1 to 3 times. Earlier, a study with 53 repeat-breeder cows indicated an improvement in pregnancy rate compared with TAI or AI at detected estrus (Son et al., 2007).

One factor that affected fertility that was not altered by embryo transfer was parity. In line with others' findings (Darwash et al., 1997; Friggens and Labouriau, 2010), primiparous cows were more fertile than multiparous cows. This was true for cows bred by TAI and

embryo transfer. It is likely that parity effects represent changes in the reproductive tract environment not correctable by embryo transfer rather than effects on the oocyte, fertilization, or embryo that could be corrected by embryo transfer.

In conclusion, the culture of embryos in BBH7 can improve blastocyst yield and survival after cryopreservation. Under these conditions of enhanced embryo competence for survival, pregnancy success in lactating cows can be improved by transfer of fresh embryos compared with TAI. Moreover, fertility did not decline when cryopreserved embryos were transferred compared with TAI, and fertility improved for transfer of vitrified embryos compared with TAI in repeat-breeder cows.

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