Embryo and cow factors affecting pregnancy per embryo transfer for multiple-service, lactating Holstein recipients

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ABSTRACT: The objective was to determine whether pregnancy success after embryo transfer (ET) during heat stress in multi-service Holstein cows depends upon characteristics of the embryo or recipient. Female embryos produced in vitro were cultured with either 0.0 (control) or 1.8 mM choline chloride and transferred fresh. Fresh embryos of undetermined breed and frozen Holstein embryos were used when experimental embryos were insufficient. Embryos were transferred 8 d after the last GnRH injection of an ovulation synchronization program. Embryo type [frozen vs. fresh, choline vs. control, unknown breed vs. (control + choline)] and characteristics of recipients (average of 190 d in milk at transfer) were evaluated. Pregnancy per ET was lower for cows receiving frozen embryos (7.0%; 3/43) than for cows receiving fresh embryos (26.7%; 32/120) but there were no differences between various types of fresh embryo. Pregnancy per ET was lower for cows diagnosed with metritis in the early postpartum period (7.1%; 2/28) than for cows without metritis (24.4%; 33/135). In conclusion, the use of frozen/thawed embryos produced in vitro and recipients which had metritis in the early postpartum period reduced the success of ET in multiple-service Holstein cows.

Key words: embryo transfer, lactating cows, metritis

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INTRODUCTION

Embryo transfer (ET) can be a valuable genetic selection tool (Kaniyamattam et al., 2017) and can enhance fertility during heat stress (Ambrose et al., 1999; Drost et al., 1999; Stewart et al., 2010; Baruselli et al., 2011) or in repeat-breeder cows (Tanabe et al., 1985; Rodriques et al., 2007; Block et al., 2010). Pregnancy success depends on characteristics of the embryo and the recipient. Embryos produced in vitro, for example, are less likely to establish pregnancy after transfer than embryos produced in vivo (Ferraz et al., 2016) and culture of embryos with embryokines can increase pregnancies per transfer (Loureiro et al., 2009; Denicol et al., 2014). Cryopreservation can lower embryo survival (Drost et al., 1999; Stewart et al.,

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2010; Ferraz et al., 2016). Success of ET also may depend upon the incidence of disease among recipients (Ferraz et al., 2016; Ribeiro et al., 2016).

Herein, we describe an experiment to evaluate embryo and cow factors that affect pregnancy per ET in cows predisposed to infertility. The study population was a group of lactating Holsteins inseminated unsuccessfully at least two times prior to enrollment. The experiment was performed during heat stress. Embryos were produced in vitro using ovaries obtained at an abattoir. The main embryo factors considered were whether the embryo was transferred fresh or after freezing/thawing and whether the culture medium used to produce embryos contained choline chloride. We tested choline chloride because it is a precursor of membrane phospholipids and the methyl donor betaine (Glier et al., 2014) and because feeding ruminally protected choline has been reported to improve fertility in Holstein cows (Ardalan et al., 2010; Zenobi et al., 2018). To date, effects of choline on preimplantation embryonic development have not been assessed. The cow factors considered were parity, milk production, days in milk, and previous occurrence in the current lactation of metritis, retained placenta, mastitis, and other diseases.

MATERIALS AND METHODS

All procedures involving cows were approved by the Animal Care and Use Committee of the University of Florida and all methods were performed in accordance with the relevant guidelines and regulations. The experiment was conducted between July 27 and October 12, 2017 at a commercial dairy farm located in north Florida (Trenton, FL; 29°35′N, 82°51′W). Meteorological conditions during the experiment were characteristic of heat stress. Average temperature-humidity index at 1500 h (calculated using the formula of Ravagnolo and Misztal, 2000) at a nearby weather station for 14-d intervals from July 14 to October 14 ranged from 80.5 to 83.3. Cows enrolled in the experiment were multiparous and primiparous lactating Holstein cows with at least two previous inseminations (3.8 ± 0.8; range of 3 to 5). The average days in milk at the time of transfer were 190 d (range 117 to 253 d). Cows were fed a total mixed ration ad libitum, were milked three times per day, and were housed in sand-bedded free-stall barns equipped with fans and sprinklers.

The reproductive management involved use of the DoubleOvsynch regimen (Souza et al., 2008) for first timed artificial insemination (AI) at 81 d in milk. Pregnancy diagnosis was performed at 33 d after AI by ultrasound. Cows diagnosed as nonpregnant received an injection of GnRH (100 µg; Fertagyl, Merck, Kenilworth, NJ), an injection of $PGF_2\alpha$ (25 mg; Lutalyse, Zoetis, Kalamazoo, MI) at day 7 and 8, a second injection of GnRH (100 µg) at day 9 (considered as day 0 of the new estrous cycle), and timed AI 24 h later. Cows enrolled in the experiment were not inseminated. Rather, ET was performed 8 d after last GnRH injection (day 17 of the protocol). Only cows with a corpus luteum (≥15 mm) detected by ultrasonography (Easy Scan; BCF Technology, Livingston, Scotland) received an embryo. Selected cows received an epidural injection of 5 mL of 2% (w/v) lidocaine (Aspen Veterinary Resources, Liberty, MO) and a single embryo was randomly transferred transcervically into the uterine horn ipsilateral to the corpus luteum. Pregnancy diagnosis was performed by transrectal ultrasonography at day 33 after last GnRH.

Each week, embryos were produced in vitro by fertilization of oocytes with X-sorted semen. Media (Ortega et al., 2017) and procedures for in vitro maturation, fertilization, and embryo culture were done as described by Siqueira and Hansen (2016). Exceptions to previously published procedures included addition of 50 µg/mL amikacin (Sigma-Aldrich, Saint Louis, MO) to prevent bacterial contamination caused by use of sexed-sorted semen. The medium for embryo culture was a proprietary medium called BBH7 (Cooley Biotech, Gainesville, FL; Block et al., 2010) or synthetic oviduct fluid bovine embryo 2 (Ortega et al., 2017).

To test the effects of choline on embryo competence for establishment of pregnancy, embryos were produced using cumulus-oocyte complexes (COC) harvested from abattoir-derived ovaries of Holstein cows located in California or Idaho (J. R. Simplot, Boise, ID). The COC were shipped overnight in a portable incubator at 38.5 °C in groups of 30 in 2 mL tubes with Simplot maturation medium. Upon arrival, COC were allowed to complete maturation for 24 h. Following maturation, COC were fertilized with X-sorted semen using one of three Holstein sires (STGenetics, Navosota, TX) and for a coincubation period of 17 to 18 h. A single sire was used each week for both treatments. Embryos were produced on different occasions (n = 7 replicates). Putative zygotes (i.e., oocytes exposed to sperm) were then cultured in groups of 30 in 50 µL drops of BBH7 culture medium at 38.5 °C in a humidified atmosphere of 5% CO_2 (v/v), 5% O_2 (v/v), and 90% N_2 (v/v). Putative zygotes were assigned randomly to culture in medium containing 1.8 mM

choline chloride (Sigma-Aldrich, Saint Louis, MO) or medium with 1.8 mM sodium chloride (Sigma-Aldrich) as a control to ensure osmolarity was the same for both treatments. The concentration of choline chloride tested was chosen because it is equivalent to the sum of concentrations of all forms of choline in blood plasma of lactating Holstein cows at 1 wk after calving (~1.3 mM; Artegoitia et al., 2014) plus the incremental increase in total choline concentration in blood plasma (0.5 mM) when Holstein cows were supplemented with ruminally protected choline during the transition period (M. Zenobi and C.R.S., personal communication). The number of embryos transferred was 48 for choline and 56 for vehicle treatments.

When the number of Holstein embryos produced for choline experiment were insufficient for the number of synchronized recipients, recipients received either a frozen/thawed, in vitro produced Holstein embryo produced by fertilization using X-sorted semen (frozen; $n = 43$) or a fresh in vitro produced embryo ($n = 16$) produced using conventional semen from beef bulls (Angus, Simental, and Polled Hereford) and oocytes from abattoir ovaries from cattle of undetermined breed. Both types of embryos were cultured in synthetic oviduct fluid bovine embryo 2. For frozen/thawed embryos, blastocysts were washed in Vigro Holding Plus Medium (Vetoquinol, Fort Worth, TX), immersed in Vigro Ethylene Glycol Freeze Plus with Sucrose (Vetoquinol, Fort Worth, TX) for 10 to 15 min at room temperature and individually loaded into a 0.25 mL straw. Subsequently, straws were frozen as described by Valente et al. (2016) using a programmable freezing machine (CL-5500, Cryologic, Victoria, Australia).

Cleavage rate was evaluated at day 3 post-insemination and blastocysts were evaluated at day 7.5 post-insemination. Blastocysts were evaluated according to the guidelines of the International Embryo Technology Society (Robertson and Nelson, 1998). Only blastocyst-stage embryos graded as 1 or 2 were transferred. Selected blastocysts were loaded into 0.25 mL straws containing transfer medium [HEPES-TALP + 10% (v/v) fetal bovine serum + 50 µM dithithreitol from Sigma-Aldrich], placed in a portable incubator (Biotherm INC-12v; Cryologic) at 38.5 °C, and transported to the farm for transfer into recipients. Frozen embryos were thawed for 5 s in air, 20 s in water at 29 °C, and transferred directly while in the original straw.

Effect of choline on percent of oocytes that cleaved after fertilization, and the percent of oocytes and cleaved embryos that developed to the blastocyst stage were analyzed by logistic regression fitted to a binomial distribution, using the GLIMMIX procedure of the Statistical Analysis System version 9.4 (SAS Institute, Cary, NC). The model included effects of choline treatment as a fixed effect and sire as a random effect. Pregnancy outcome (pregnant or nonpregnant) on day 33 after presumptive ovulation also was considered a binomial variable and analyzed by logistic regression using the GLIMMIX procedure of SAS. Factors evaluated from recipients were parity (≤2 vs. >2), days in milk (≤192 vs. >192 d), milk production at the test day closest to ET (≤40.8 vs. >40.8 kg/d), diagnosis of metritis (presence of foul-smelling, watery vaginal discharge, with or without fever, during the first 21 d in milk of the current lactation), retained placenta, mastitis diagnosed within 30 d of ET, and other diseases (ketosis, digestive and respiratory diseases) diagnosed at any point between calving and pregnancy diagnosis. Embryo type included fresh control, fresh choline, embryos of unknown breed, and frozen embryos. Sources of variation with a *P*-value greater than 0.100 were excluded from the final model. Orthogonal contrasts were used to investigate the effects of embryo type [frozen vs. others, choline vs. control, unknown breed vs. (control + choline)].

To further examine the effect of previous diagnosis of metritis on pregnancy outcome, an additional analysis was conducted using fresh embryos only (to avoid potential confounding effect of cryopreservation). The effect of previous diagnosis of metritis was analyzed using a one-tailed Wilcoxon's test with the NPAR1WAY procedure of SAS.

RESULTS AND DISCUSSION

The two major determinants of pregnancy success after ET were whether the embryo was fresh or frozen ($P = 0.007$) and whether the recipient had been previously diagnosed with metritis ($P = 0.059$; Fig. 1). Recipients that received a frozen embryo had a lower pregnancy per ET (3/43; 7.0%) than recipients receiving a fresh embryo (32/120; 26.7%). Lower pregnancy success after transfer of frozen/thawed embryos was expected because of the often-reported poor survival of in vitro produced embryos frozen using ethylene glycol (Ambrose et al., 1999; Drost et al., 1999) or glycerol (Wurth et al., 1994). There is, however, great variation in pregnancy success following transfer of frozen embryos produced in vitro, probably due to differences in molecular characteristics of in vitro produced embryos between laboratories. For example,

40.2% of in vitro produced embryos cryopreserved in ethylene glycol established pregnancy after transfer (Valente et al., 2016). Pregnancy per ET also was lower for recipients that had been diagnosed previously with metritis (2/28; 7.1%) compared with those that had not been diagnosed with metritis (33/135; 24.4%). The negative effect of previous diagnosis of metritis on pregnancy success was observed regardless of whether a recipient received a fresh or frozen embryo (Fig. 1). Further analysis of pregnancy outcomes for cows receiving a fresh embryo indicated that pregnancy per ET was lower ($P = 0.027$) for recipients that had been diagnosed previously with metritis (2/21; 9.5%) compared with those that had not been diagnosed with metritis (30/99; 30.3%).

Ribeiro et al. (2016) reported that diagnosis of uterine or nonuterine diseases in the early postpartum period was associated with reduced pregnancy success after the first breeding by either AI or ET. However, all recipients in the present study had at least two previous unsuccessful services before enrollment in ET and were further in the postpartum period (average = 190 d in milk) than the cows in the study of Ribeiro et al. (2016). Thus, it is likely that metritis can either create a uterine environment hostile for the establishment of pregnancy for weeks after diagnosis or else that undiagnosed uterine disease was still present at the time of ET. One implication of these results is that use of ET to improve fertility of repeat-breeder cows (Tanabe et al., 1985; Rodriques et al., 2007; Block et al., 2010) is likely to depend on the cause of the inherent fertility and may not be effective for cows which experienced metritis.

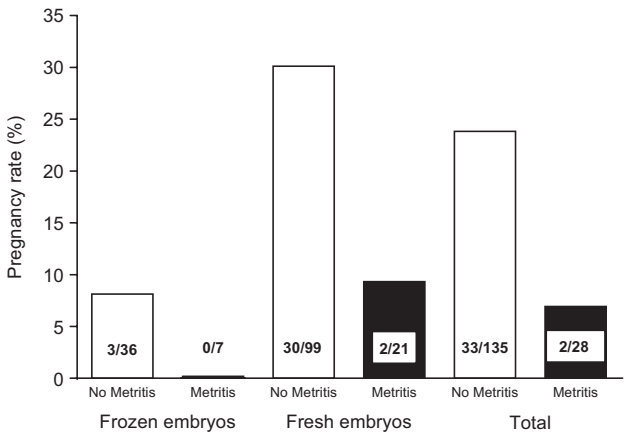

Figure 1. Effects of previous diagnosis of metritis in the recipient and cryopreservation of the embryo on pregnancy per embryo transfer. Pregnancy outcome was affected by the comparison of fresh vs. frozen ($P = 0.007$) and whether the recipient had been previously diagnosed with metritis ($P = 0.059$).

There were no effects ($P > 0.10$) on pregnancy outcome when other recipient characteristics were examined (parity, days in milk, milk production, retained placenta, mastitis, and other diseases), possibly due to the small number of observations. In a study involving over 10,000 transfers, pregnancy per ET was lower for multiparous cows than primiparous cows, lower for cows diagnosed with metritis, slightly lower for recipients which experienced calving problems and not associated with milk yield at ET (Ferraz et al., 2016).

As shown in Table 1, there was no effect of addition of choline chloride to the culture medium on the percent of oocytes that cleaved after fertilization ($P = 0.290$), percent of oocytes becoming blastocysts ($P = 0.490$), or percent of cleaved embryos that developed to the blastocyst stage ($P = 0.326$). Pregnancy per ET was not affected ($P = 0.284$) by the presence of choline in culture medium (Table 2). Numerically, pregnancy per ET was slightly higher for cows receiving an embryo treatment with choline but the small number of cows precludes definitive conclusions.

The absence of effects of choline on the competence of embryos to develop to blastocyst stage was striking because choline is a precursor of membrane phospholipids and its metabolite betaine is involved in DNA methylation (Glier et al., 2014). The preimplantation embryo undergoes extensive cell proliferation and the eight-cell stage embryo undergoes re-methylation of DNA (Dobbs et al., 2013), making phospholipids and methyl groups critical for its development and pregnancy establishment. Perhaps stores of choline and its metabolites in the oocyte or de novo synthesis are adequate for the embryo to meet its needs. In mice, betaine is synthesized during oocyte development and is present in the preimplantation embryo (McClatchie et al., 2017). It also has been observed that transcripts

Table 1. Effect of choline supplementation of embryo culture medium on in vitro production of blastocysts[1]

Variable	Control	Choline	*P*-value
Oocytes undergoing cleavage, %	72.9 ± 1.3	69.2 ± 1.3	0.290
Oocytes becoming blastocyst, %	17.2 ± 1.1	18.8 ± 1.1	0.490
Cleaved embryos becoming a blastocyst, %	24.6 ± 1.5	28.8 ± 1.6	0.326

[1]Data are least-squares means ± SEM of results from seven replicates representing 1,335 (control) or 1,332 (choline) cumulus-oocyte complexes (choline).

Table 2. Pregnancy per embryo transfer (fraction of cows pregnant) for recipients receiving fresh embryos produced in control medium or medium containing 1.8 mM choline chloride

Treatment	Metritis	No metritis	Total
Control	0% (0/11)	24.4% (11/45)	19.6% (11/56)
Choline	25% (2/8)	30.0% (12/40)	29.2% (14/48)

coding for enzymes involved in betaine and phosphatidylcholine synthesis are present during the preimplantation period in bovine embryos (Jiang et al., 2014). In the present study, choline chloride was used to provide choline to the embryo but the main metabolite in the blood plasma of dairy cows is phosphatidylcholine (Artegoitia et al., 2014). Perhaps, phosphatidylcholine is utilized differently by the embryo than choline chloride.

In conclusion, the use of frozen embryos produced in vitro and recipients which had metritis in the early postpartum period reduced the success of ET during the heat stress season in multiple-service Holstein cows. The negative effect of metritis on the success of ET was still present after long period from the diagnosis in the early postpartum.

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Conflict of interest statement: P.J.H. has an ownership interest in Cooley Biotech, manufacturers of BBH7 culture medium.

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