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Effect of transfer of one or two in vitro-produced embryos and post-transfer administration of gonadotropin releasing hormone on pregnancy rates of heat-stressed dairy cattle

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Abstract

Pregnancy rates following transfer of an in vitro-produced (IVP) embryo are often lower than those obtained following transfer of an embryo produced by superovulation. The purpose of the current pair of experiments was to examine two strategies for increasing pregnancy rates in heat stressed, dairy recipients receiving an IVP embryo. One method was to transfer two embryos into the uterine horn ipsilateral to the CL, whereas the other method involved injection of GnRH at Day 11 after the anticipated day of ovulation. In Experiment 1, 32 virgin crossbred heifers and 26 lactating crossbred cows were prepared for timed embryo transfer by being subjected to a timed ovulation protocol. Those having a palpable CL were randomly selected to receive one ($n = 31$ recipients) or two ($n = 27$ recipients) embryos on Day 7 after anticipated ovulation. At Day 64 of gestation, the pregnancy rate tended to be higher ($P = 0.07$) for cows than for heifers. Heifers that received one embryo tended to have a higher pregnancy rate than those that received two embryos (41% versus 20%, respectively) while there was no difference in pregnancy rate for cows that received one or two embryos (57% versus 50%, respectively). Pregnancy loss between Day 64 and 127 only occurred for cows that received two embryos (pregnancy rate at Day 127 = 17%). Between Day 127 and term, one animal (a cow with a single embryo) lost its pregnancy. There was no difference in pregnancy rates at Day 127 or calving rates between cows and heifers, but females that received two embryos had lower Day-127 pregnancy rates and calving rates than females that received one embryo ($P < 0.03$). Of the females receiving two embryos that calved, 2 of 5 gave birth to twins. For Experiment 2, 87 multiparous, late lactation, nonpregnant Holstein cows were synchronized for timed embryo transfer as in Experiment 1. Cows received a single embryo in the uterine horn ipsilateral to the ovary containing the CL and received either 100 μg GnRH or vehicle at Day 11 after anticipated ovulation (i.e. 4 days after embryo transfer). There was no difference in pregnancy rate for cows that received the GnRH or vehicle treatment (18% versus 17%, respectively). In conclusion, neither unilateral transfer of two embryos nor administration of GnRH at Day 11 after anticipated ovulation improved pregnancy rates of dairy cattle exposed to heat stress.

Keywords: Embryo transfer; Twinning; GnRH; Dairy cattle

1. Introduction

The in-vitro-produced (IVP) embryo is different from the embryo produced in vivo in terms of morphology [1–3], gene expression [4–6], metabolism [7], and incidence of chromosomal abnormalities [3,8]. Not surprisingly,

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pregnancy rates achieved following transfer of an IVP embryo are often less than what is obtained following transfer of an embryo produced by superovulation, and calves born as the result of in vitro production are more likely to experience developmental defects [9]. Problems associated with the transfer of IVP embryos have limited the realization of the potential of these embryos for enhancing genetic improvement and reproductive performance of lactating dairy cattle [10,11].

One method that might be useful for increasing pregnancy rates in dairy cattle recipients that receive an IVP embryo is to transfer two embryos into the uterine horn ipsilateral to the CL. Such a treatment might increase pregnancy rate, because the likelihood is increased that the cow receives at least one embryo competent for sustained development. In addition, the transfer of two embryos into the ipsilateral uterine horn is likely to increase the amounts of interferon-τ and other embryonic signaling molecules in the uterus needed to maintain pregnancy and prevent luteolysis. Co-transfer of embryonic vesicles to increase trophoblastic signals has been reported to increase pregnancy rates in embryo transfer recipients [12]. For the current experiment, both embryos were transferred into the uterine horn ipsilateral to the CL because of the requirement for the antiluteolytic signal in cattle to be locally administered [13,14]. In a recent study with a small number of transfers ($n = 10–28$ recipients), there was a tendency for higher calving rate for recipients that received two embryos in the uterine horn ipsilateral to the CL as compared to recipients that received one embryo [15]. Anderson et al. [16] found a tendency for pregnancy rates to be higher in cows that received two embryos in the same uterine horn (unilateral transfer) than for cows that received two embryos distributed in both uterine horns (bilateral transfer); the opposite was true for heifers. In other studies, transfer of embryos to create two pregnancies in the uterine horn ipsilateral to the CL has produced a similar pregnancy rate as bilateral twins and single pregnancies [17,18] or reduced pregnancy rate as compared to bilateral transfer [19].

Another treatment that has potential for increasing pregnancy rates in embryo transfer recipients is injection of GnRH at Day 11 after the anticipated day of ovulation; it increased pregnancy rates in heat-stressed, lactating cows following insemination [20,21] and embryo transfer [22]. Treatment of GnRH or its analogues at Days 11–12 of the estrous cycle has been reported to increase progesterone secretion [21,23] and inhibit function of the dominant follicle [23,24] to possibly delay luteolysis.

The purpose of the current pair of experiments was to examine the effectiveness of unilateral transfer of twin embryos and treatment with GnRH at Day 11 after the anticipated day of ovulation for increasing pregnancy rates in dairy cattle recipients that received an IVP embryo. Experiments were performed during periods of heat stress, because embryo transfer offers benefits as a method for increasing pregnancy rate as compared to AI in females subjected to heat stress [10].

2. Materials and methods

2.1. Experiment 1—single or twin transfer of IVP embryos into crossbred dairy recipients

The experiment was conducted at a commercial dairy located in Santa Cruz, Bolivia (17°48′S, 63°10′W) from November to December, 2004. Data on minimum and maximum air temperatures during the experiment collected by Servicio Nacional de Meteorologia e Hidrologia (http://www.senamhi.gov.bo/meteorologia/) for Santa Cruz are presented in Fig. 1. Females receiving embryos included 32 virgin crossbred heifers sired by Simmental, Gyr, or Brown Swiss bulls and Holstein or

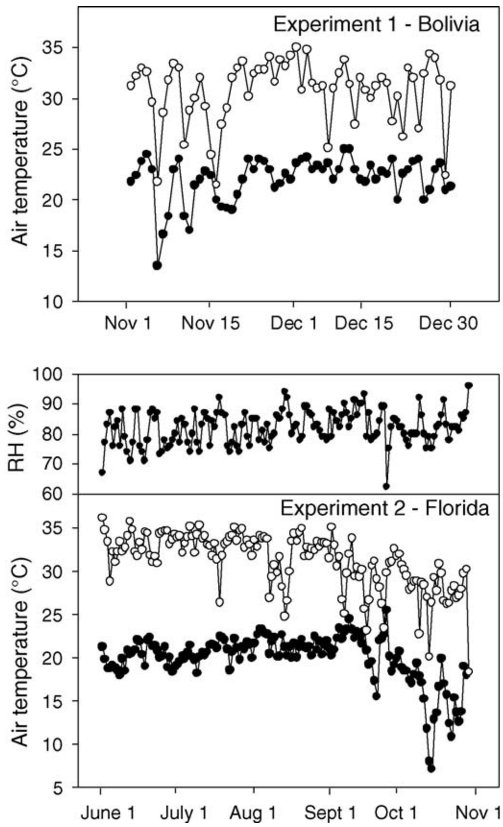

Fig. 1. Maximum (open circles) and minimum (closed circles) daily air temperatures and relative humidities (RH) during the experiments.

Holstein crossbred dams and 26 lactating, crossbred cows with the proportion of Holstein varying from 1/2 to 15/16. The heifers ranged in age from 363 to 2070 days (mean = 850 days and median = 664 days; S.D. = 421 days) and ranged in weight from 247 to 430 kg (mean = 310 kg and median = 288 kg; S.D. = 52.3 kg). Animals were maintained on grass pasture until 2 weeks prior to the start of the synchronization program when they also received a supplement of 6 kg/head/day of spent brewers' grain. The cows ranged in age from 820 to 4075 days (mean = 2083 days and median = 1670 days; S.D. = 986 days), were maintained on grass pasture, and received 11 kg of brewers' grains and 2 kg of a soybean-based concentrate mixture before each milking. Cows were milked two times per day and ranged from 110 to 417 days in milk (mean = 190 days and median = 170 days; S.D. = 75 days). Milk yield per day across all days of lactation ranged from 5.9 to 21.1 kg/day (mean = 12.5 kg/day and median = 12.6 kg/day; S.D. = 3.8 kg/day).

Recipients were synchronized for timed embryo transfer using a modified OvSynch protocol [25], with the inclusion of a controlled intravaginal drug-releasing device (EAZI-BREED CIDR® insert, 1.38 g of progesterone; Pfizer Animal Health, New York, NY, USA). On Day −10 (Day 0 equals the day of anticipated ovulation), females were given 100 μg (i.m.) of GnRH (1 mL of Profertil®; Tortuga Cia. Zootécnica Agrária, São Paulo, Brazil) and an intravaginal progesterone-releasing device insert that had been used one time previously. On Day −3, CIDR devices were removed and females received 150 μg (i.m.) of $PGF_{2\alpha}$ (2 mL of Prostaglandina Tortuga; Tortuga Cia. Zootécnica Agrária). On Day 0, 100 μg (i.m.) of GnRH was given. Behavioral symptoms of estrus were monitored about five times daily for 3 days following CIDR removal and $PGF_{2\alpha}$ injection. On Day 6 after anticipated ovulation, all females, including those not seen in estrus, were subjected to transrectal ultrasonography for the presence of a CL, using an Aloka 210 ultrasound unit equipped with a 5 MHz linear-array probe (Aloka, Wallingford, CT, USA). A group of females having a CL ($n = 32$ heifers and $n = 26$ cows) were randomly selected within recipient type (heifers or cows) to receive one ($n = 31$ females) or two ($n = 27$ females) embryos on Day 7 after anticipated ovulation. For embryo transfer, an epidural block of 5 mL of lidocaine hydrochloride (2%, w/v; Sparhawk Laboratories Inc., Lenexa, KS, USA) was administered to each recipient, and one or two IVP embryos were deposited into the uterine horn ipsilateral to the ovary containing the CL. One technician conducted all transfers.

A total of 85 blastocysts (72 at Day 7 after insemination and 13 at Day 8 after insemination) were transferred in this experiment. Of these, six were produced by Transova (Sioux City, IA, USA) using Holstein oocytes and a Holstein sire and were cultured in Synthetic Oviductal Fluid (SOF) medium. Embryos were shipped overnight in a portable incubator to Gainesville, FL, USA on Day 4 after insemination. Embryos were transferred to fresh microdrops of a modified SOF [26] prepared by Specialty Media (Phillipsburg, NJ, USA) and cultured at 38.5 °C in a humidified atmosphere of 5% O_2 and 5% (v/v) CO_2 (balance N_2). The remainder were produced using oocytes obtained from ovaries of a variety of breeds collected at a local abattoir, located at a travel distance of approximately 1.5 h from the Gainesville laboratory. Procedures, reagents, and media formulation for oocyte maturation, fertilization, and embryo culture were as previously described [27], with some modifications. Cumulus-oocyte complexes were matured for approximately 22 h at 38.5 °C in an atmosphere of 5% (v/v) CO_2 in humidified air and then inseminated with a cocktail of Percoll-purified spermatozoa from three different bulls of various breeds. At 8–12 h post-insemination (hpi), putative zygotes were denuded of cumulus cells by suspension in Hepes-TALP medium (Caisson, Rexburg, ID, USA) containing 1000 units/mL hyaluronidase type IV (Sigma, St Louis, MO, USA) and vortexed in a microcentrifuge tube for 5 min. Presumptive zygotes were then placed in groups of ~30 in 50 μL microdrops of KSOM-BE2 [28] (Caisson, Rexburgh, ID, USA) at 38.5 °C in an atmosphere 5% (v/v) CO_2 in air.

Regardless of method of production, embryos greater than 16 cells in appearance were collected at 13:00 h on Day 6 or 7. Embryos were placed in groups of 21–65 in a 2 mL cryogenic vial (Nalge Company, Rochester, NY, USA) filled to the top with KSOM-BE2 that was pre-warmed and equilibrated in 5% (v/v) CO_2 in air. Embryos produced by Transova were kept separately from those produced using ovaries from the local abattoir. Vials containing embryos were placed in a portable incubator (Minitube of America, Verona, WI, USA) that had been pre-warmed to 39 °C for 24 h prior to use. Embryos were shipped by air and arrived at Santa Cruz de la Sierra, Bolivia, at 11:00 h the next day (Day 7 or 8 after in vitro insemination) and transported by ground to the farm.

Embryos were transferred over a time span from 13:00 and 20:00 h. One or two embryos were loaded into 0.25 mL straws in Hepes-TALP (Caisson) containing 10% (v/v) bovine steer serum (Pel-Freez, Rogers, AR, USA) and 100 μM 2-mercaptoethanol (Sigma-

Aldrich, St. Louis, MO, USA). Embryos were transferred to recipients that were palpated the day before and had a detectable CL. Recipients were randomly assigned to receive one or two embryos, and all embryos were transferred into the ipsilateral horn to the CL. Pregnancy diagnosis was performed by transrectal palpation at Days 64 and 127 post-transfer, and the number of fetuses was recorded on Day 127. Data collected at calving included length of gestation (with the day of transfer being considered Day 7 of gestation), occurrence of dystocia (defined as needing assistance), sex, weight and viability of each calf, and occurrence of retained placenta (failure of the placenta to be expelled within 12 h after calving). Calf survival until Day 7 of age was also recorded.

2.2. Experiment 2—administration of GnRH on Day 11 after anticipated ovulation in lactating recipients that received an IVP embryo

This study took place at a commercial dairy located in Bell, FL, USA (29°45′N 82°51′W) from June to October, 2004. Data on minimum and maximum air temperatures and average relative humidity collected by the Florida Automated Weather Service (http://fawn.ifas.ufl.edu) for Alachua, FL, USA are presented in Fig. 1. A total of 87 multiparous, lactating Holstein cows in late lactation were used as recipients. Cows were fed a total mixed ration to meet or exceed requirements recommended for lactating dairy cows, milked three times a day, and received bovine somatotropin (500 mg sometribove zinc; Posilac®, Monsanto, St. Louis, MO, USA) according to manufacturer's directions. Cows were housed in a dry lot with access to a permanent shade structure without fans or sprinklers and with access to a cooling pond.

Cows were prepared for embryo transfer in groups of 6–18; a total of 10 replicates were completed. To synchronize recipients for timed embryo transfer, cows received 100 μg (i.m.) of GnRH (2 mL of Cystorelin®;Merial Limited, Iselin, NJ, USA), on Day −10; 25 mg (i.m.) of $PGF_{2\alpha}$, on Day −3; and 100 μg (i.m.) of GnRH, on Day 0 (i.e. the day of anticipated ovulation). On Day 7 after anticipated ovulation, all cows were palpated per rectum for the presence of a CL. Cows that had a palpable CL received an epidural block of 5 mL of lidocaine (2%, w/v), and a single embryo was transferred to the uterine horn ipsilateral to the ovary containing the CL. Recipients were randomly assigned to receive 100 μg (i.m.) of GnRH or vehicle (9 mg/mL of benzyl alcohol and 7.47 mg/mL of sodium chloride in water) on Day 11 after anticipated ovulation.

The embryos used for transfer were produced in the Gainesville laboratory using oocytes of various breeds and a pool of semen from three bulls of various breeds as described for Experiment 1. A different pool of semen was used for each replicate. Presumptive zygotes were cultured in groups of ~30 in 50 μL microdrops of modified SOF [26] containing 100 ng/mL of insulin-like growth factor-1 (Upstate Biotechnology, Lake Placid, NY, USA). Embryos were cultured at 38.5 °C in a humidified atmosphere of 5% (v/v) O_2 and 5% (v/v) CO_2 with the balance N_2. On Day 7 after insemination, blastocysts were harvested and transported to the farm in 2 mL cryogenic vials (20–25 embryos/tube) filled to the top with pre-warmed Hepes-TALP. Tubes containing embryos were placed in a portable incubator (Minitube of America) that had been pre-warmed to 39 °C for 24 h prior to use. Embryos were transported to the farm and loaded in 0.25 mL straws prior to transfer into recipients. Pregnancy was diagnosed by transrectal palpation on Days 45–53 after anticipated ovulation.

2.3. Statistical analysis

Categorical data were analyzed by logistic regression using the LOGISTIC procedure of SAS for Windows (Version 9, SAS Institute Inc., Cary, NC, USA) with a backward stepwise logistic model. Variables were continuously removed from the model by the Wald statistic criterion if the significance was greater than 0.2. The full statistical model for Experiment 1 included treatment (one embryo or two embryos), parity (cows versus heifers), estrus (observed in estrus versus not observed) and treatment × parity on pregnancy rate, pregnancy loss, calving rate, calf mortality and twinning rate. The only variable in the final mathematical model for Experiment 2 was GnRH treatment, as other effects (replicate and replicate × treatment) were not significant. The adjusted odds ratio estimates and the 95% Wald confidence intervals (CI) from logistic regression were obtained for each variable that remained in the final statistical model following the backward elimination. Data were also analyzed with the GENMOD procedure of SAS to determine the significance of each effect that remained in the reduced model; *P*-values for logistic regression analyses reported in the tables are derived from these analyses. Data for gestation length and calf birth weight were analyzed by analysis of variance using Proc GLM. The full statistical model included the effects of treatment, parity and treatment × parity. The χ^2-test was used to determine whether the sex ratio of calves differed from the expected 1:1 ratio.

3. Results

3.1. Experiment 1—single or twin transfer of IVP embryos

3.1.1. Pregnancy and calving rates

Data are summarized in Table 1. At Day 64 of gestation, the pregnancy rate tended to be higher ($P = 0.07$) for cows than for heifers. While there were no significant effects of number of embryos transferred or parity × number transferred, heifers that received two embryos tended to have lower pregnancy rates than those that received a single embryo (20% for two embryos versus 41% for one embryo), whereas there was no difference in pregnancy rate due to number of embryos transferred to cows (50% for two embryos versus 57% for one embryo).

Pregnancy losses between Day 64 and 127 occurred in one group only—cows receiving two embryos. In that group, pregnancy rate was 50% at Day 64 but decreased to 17% at Day 127. There was no difference in pregnancy rates at Day 127 between cows and heifers, but recipients that received two embryos had lower pregnancy rates (17% for cows and 20% for heifers) than recipients that received one embryo (57% for cows and 41% for heifers, $P < 0.03$).

Pregnancy loss after Day 127 occurred in one female only. In particular, a cow receiving a single embryo gave birth to a stillborn calf at 251 days of gestation. Similar to pregnancy rate at Day 127, there was no difference in calving rate between cows and heifers, but recipients that received two embryos had lower calving rates (17% for cows and 20% for heifers) than recipients that received one embryo (50% for cows and 41% for heifers, $P < 0.03$).

Estrus was detected at 24, 48 or 72 h after prostaglandin injection in 21/32 heifers (eight at 24 h after injection and 13 at 48 h) and 19/26 cows (one at 24 h after injection, 14 at 48 h, and 4 at 72 h). While not statistically different ($P = 0.11$), there was a tendency for pregnancy rates to be lower for animals not detected in estrus. For example, pregnancy rates at Day 127 for animals receiving one embryo was 55% (11/20) for animals in estrus versus 36% (4/11) for animals not observed in estrus. Pregnancy rates at Day 127 for animals receiving two embryos were 25% (5/20) for animals in estrus versus 0% (0/7) for animals not observed in estrus.

3.1.2. Characteristics of gestation, parturition, and calves

Gestation length was affected by recipient type × number of embryos transferred ($P < 0.05$; Table 2). For cows, gestation length was slightly longer for those receiving one embryo as compared to those receiving two embryos, whereas the opposite was true for heifers.

Table 1
Effect of recipient type and number of embryos transferred per recipient on pregnancy rates and losses in cattle

Recipient type	Pregnancy rate (Day 64 of gestation)[a,b]	Pregnancy rate (Day 127 of gestation)[a,c]	Pregnancy loss (Days 64–127 of gestation)[d]	Calving rate[e,f]	Pregnancy loss (Day 127 to calving)[g]
Lactating cow, single embryo	8/14 (57%)	8/14 (57%)	0/8 (0%)	7/14 (50%)	1/8 (13%)[h]
Lactating cow, two embryos	6/12 (50%)	2/12 (17%)	4/6 (66%)	2/12 (17%)	0/2 (0%)
Nulliparous heifer, single embryo	7/17 (41%)	7/17 (41%)	0/7 (0%)	7/17 (41%)	0/7 (0%)
Nulliparous heifer, two embryos	3/15 (20%)	3/15 (20%)	0/3 (0%)	3/15 (20%)	0/3 (0%)

[a] Data are the proportion of animals pregnant of those that received embryos and, in parentheses, the percent pregnant.
[b] Logistic regression indicated effect of recipient type ($P = 0.07$). The odds ratio estimate was 0.38 (heifer/cow) (95% Wald CI = 0.13, 1.14; Wald χ^2 statistic = 2.96, $P = 0.08$).
[c] Logistic regression indicated an effect of number of embryos transferred ($P < 0.03$). The odds ratio estimate was 4.13 (one embryo/two embryos) with a 95% Wald CI of 1.243, 13.690. Wald χ^2 statistic = 5.36; $P < 0.03$).
[d] Data are the proportion of pregnant recipients at Day 64 that lost their pregnancy by Day 127 of gestation and, in parentheses, the percent pregnancy loss.
[e] Data are the proportion of animals that calved of those that received embryos and, in parentheses, the percent pregnant.
[f] Logistic regression indicated an effect of number of embryos transferred ($P < 0.03$). The odds ratio estimate was 3.62 (one embryo/two embryos) with a 95% Wald CI of 1.090, 12.047. Wald χ^2 statistic = 4.41; $P < 0.04$).
[g] Data are the proportion of pregnant recipients at Day 127 that lost their pregnancy before calving and, in parentheses, the percent pregnancy loss.
[h] One cow expelled a stillborn calf at Day 251 of gestation.

Table 2
Effect of recipient type and number of embryos transferred per recipient on characteristics of pregnancy and parturition in cattle

Recipient type	Gestation length (days)[a]	Twin pregnancies[b]	Dystocia[c]	Retained placenta[d]
Lactating cow, single embryo	282 ± 3	0/7 (0%)	2/7 (29%)	4/7 (57%)
Lactating cow, two embryos	274 ± 5	1/2 (50%)	0/2 (0%)	1/2 (50%)
Nulliparous heifer, single embryo	276 ± 3	0/7 (0%)	1/7 (14%)	5/7 (71%)
Nulliparous heifer, two embryos	284 ± 4	1/3 (33%)	1/3 (33%)	2/3 (67%)

[a] Data are least-squares mean ± S.E.M. Gestation length was affected by recipient type × number of embryos transferred ($P < 0.05$).
[b] Data are the proportion of pregnancies in which twin calves were born and, in parentheses, the percent pregnant. Logistic regression indicated an effect of number of embryos transferred ($P < 0.02$).
[c] Data are the proportion of pregnancies in which dystocia was recorded at birth and, in parentheses, the percent cows experiencing dystocia.
[d] Data are the proportion of cows calving that experienced retained placenta and, in parentheses, the percent cows experiencing retained placenta.

Table 3
Effect of recipient type and number of embryos transferred per recipient on characteristics of calves born

Recipient type	Sex ratio (M:F)[a]	Calf birth weight (kg)[b]	Calf mortality at birth[c]	Calf mortality to Day 7 of age[d]
Lactating cow, single embryo	5:3[e]	34 ± 3	0/7 (0%)	0/7 (0%)
Lactating cow, two embryos	2:1	25 ± 5	0/3 (0%)	0/3 (0%)
Nulliparous heifer, single embryo	4:3	26 ± 3	1/7 (14%)[f]	0/6 (0%)
Nulliparous heifer, two embryos	4:0	25 ± 5	3/4 (75%)[g]	0/1 (0%)

[a] The overall sex ratio of 15 male and 7 females tended to be different ($P < 0.10$) than the expected 1:1 ratio.
[b] Data are least-squares mean ± S.E.M.
[c] Data are the proportion of calves that were born dead and, in parentheses, the percent born dead.
[d] Data are the proportion of calves born alive that died before Day 7 of live and, in parentheses, the percent death before Day 7.
[e] Data includes the stillborn calf at 251 days of gestation.
[f] One calf was dead at birth from a cow not experiencing dystocia.
[g] One heifer had twin fetuses and both were born dead as a result of complications with calving. The other two heifers gave birth to a single calf. One calf was born alive and the other was born dead as a result of complications with calving.

Two of 5 females calving that received two embryos produced twin calves. There was no significant effect of recipient type or number of embryos transferred on dystocia or incidence of retained placenta (Table 2). Sex ratio (including the one stillborn calf) was in favor of males, with 15 males compared to seven female calves born (68% male; Table 3). This ratio tended to be different from the expected 1:1 ratio ($P < 0.10$).

While there were no significant differences, there was a tendency for calf mortality at birth to be greater for heifers receiving two embryos than for other groups (Table 3). None of the cows lost their calf at birth and only 1 of 7 heifers receiving a single embryo experienced calf death at birth. In contrast, 2 of 3 heifers receiving two embryos experienced calf loss. One heifer had twin fetuses and both were born dead as a result of complications with calving. Another heifer gave birth to a single calf that was born dead as a result of complications with calving. The calf from the third heifer was born alive. All calves born alive were still alive 7 days later.

3.2. Experiment 2—administration of GnRH on Day 11 after anticipated ovulation

Administration of GnRH at Day 11 after anticipated ovulation had no effect ($P > 0.10$) on pregnancy rates. Recipients treated with GnRH had a pregnancy rate of 17.8% (8/45), whereas recipients that received placebo had a pregnancy rate of 16.7% (7/42). The odds ratio was 1.08 with 95% Wald confidence interval of 0.23 and 3.30.

4. Discussion

The purpose of the experiments described here was to examine two strategies for increasing pregnancy rates in heat-stressed dairy recipients that receive an IVP embryo. Neither approach, transferring two embryos into the uterine horn ipsilateral to the CL or injection of GnRH at Day 11 after anticipated ovulation, increased pregnancy rates.

In Experiment 1, the transfer of two embryos into recipients led to pregnancy loss; this loss occurred earlier for heifers than for cows. As early as Day 64 of gestation, there was a distinct difference in pregnancy rate between heifers that received one or two embryos. Among cows, in contrast, there were no differences in pregnancy rate at this stage of gestation between recipients that received one or two embryos. By Day 127, however, cows that received two embryos experienced substantial mid-to-late fetal loss and pregnancy rate and subsequent calving rate was lower for this group than for cows that received a single embryo.

The most likely explanation for the increased frequency of pregnancy loss in recipients receiving two embryos is uterine crowding, with the effects of crowding occurring sooner in gestation for nulliparous animals than for multiparous animals. Similar results were obtained in another study [16]. In that study, calving rates and twinning rates were similar for cow recipients, regardless of whether twin transfers were performed via bilateral or unilateral placement. For heifers, in contrast, calving rate and twinning rate was lower for unilateral twin transfers than for bilateral transfers. Using heifers, Rowson et al. [19] also found lower embryonic survival rates and twinning rates for recipients of unilateral twin transfers than for recipients of bilateral transfers.

It is evident, however, that herds of cattle vary in their uterine capacity. Thus, there were no differences in pregnancy success between recipients of twin embryos placed unilaterally or bilaterally for heifers [17,18] or cows [17]. Similarly, embryonic survival rate for beef cows selected for twinning was similar for those having unilateral or bilateral multiple ovulations [29]. In lactating dairy cows, in contrast, the likelihood of a twin pregnancy resulting from multiple ovulation going to term was higher if ovulations occurred bilaterally than if unilateral ovulations occurred [30]. Perhaps, identification of the biological processes controlling uterine capacity will lead to new approaches for increasing the efficacy of producing twins in cattle.

In an earlier study, administration of GnRH at Day 11 after anticipated ovulation tended to increase pregnancy and calving rates in lactating Holstein recipients [22]. The management of these cows was similar to those in Experiment 2. In both studies, recipients were exposed to heat stress and received an IVP embryo using a timed embryo transfer protocol. Effectiveness of treatment with GnRH or its analogues at 11–12 days after estrus for inseminated cows has yielded variable results, as some reports indicated a positive effect [20,21], whereas others indicated no effect [23]. One factor that could influence the effectiveness of GnRH treatment at Day 11 is the number of follicular waves that a female experiences during an estrous cycle. Females with estrous cycles characterized by three follicular waves have larger second-wave dominant follicles at Day 11 than females with two-wave cycles [24,31,32]. That a follicle must reach 10 mm in diameter to ovulate in response to LH [33], the preponderance of cycle type (two-wave versus three-wave) within a herd may determine effectiveness of GnRH treatment at Day 11. Finally, it remains possible that failure to observe an effect of GnRH treatment was because the number of animals per group was low. The pitfalls associated with interpretation of experiments with low numbers has been discussed [34] and could be responsible for the variation in results for trials to test effects of GnRH on pregnancy rates in embryo transfer recipients.

Estrus is difficult to detect in lactating dairy cows because of the short duration of estrus and the large proportion of cows that do not display intense mounting activity [35]. This problem, which is exacerbated by heat stress [36], makes embryo transfer in lactating cows inefficient if recipient selection is based solely on estrus detection. The first report of a timed embryo transfer protocol, where ovulation was synchronized using an OvSynch protocol, was by Ambrose et al. [37]. The suitability of timed embryo transfer as a method for preparing recipients was demonstrated in Experiment 1 because calving rates were 50 and 41% for cow and heifer recipients that received a single embryo, respectively. Similarly, using beef recipients, a pregnancy rate of 49% was achieved using timed embryo transfer [39]. In contrast, pregnancy rate at Day 45 of gestation in Experiment 2 was only 17%. Low pregnancy rates have been reported in other studies with timed embryo transfer using lactating, heat-stressed recipients with pregnancy rates at ~45 days of gestation following timed embryo transfer ranging from 11 to 26% [22,37,38]. The reason for the differences in pregnancy rates between Experiment 1 and 2 cannot be deduced because of the large number of variables between studies including nutrition, housing, milk yield, stage of lactation, breed, and synchronization protocol.

Despite the effectiveness of timed embryo transfer, there was a tendency for pregnancy rates in Experiment 1 to be higher for those recipients detected in estrus. Most of the animals not detected in estrus likely ovulated after the last GnRH injection because embryos were only transferred to recipients with a detectable CL. Nonetheless, some cows in this group probably were not synchronized with respect to predicted ovulation time.

Transfer of IVP embryos has been associated with large calf syndrome, increased rates of fetal loss, sex ratio skewed towards the male, and increased rates of dystocia and calf mortality (see [40–42] for review). There are also reports of prolonged gestation length [43,44]. In Experiment 1, most characteristics of the fetus and calf that were measured in females receiving one embryo were within normal ranges including gestation length, rates of fetal loss, calf birth weight, and calf survival at birth and within the first 7 days of age. The incidence of dystocia among females receiving one calf was 21% and it is difficult to determine whether this value is high because of the particular mating combinations used (embryos of diverse genotypes transferred into females of several different genotypes). In a study with Holsteins bred by artificial insemination, the frequency of difficult births ranged from 6 to 18% [45].

One abnormality identified was a skewed sex ratio, with 68% of the calves being male. While previous work suggests that the altered sex ratio among IVP embryos is due to toxic effects of concentrations of glucose in excess of 1 mM on female embryos [46], the concentration of glucose in the medium used for culture here (KSOM-BE2) contains only 0.2 mM glucose [28]. Others have found a tendency for male embryos to become blastocysts sooner in development when cultured in KSOM than female embryos [47]. Differences in sex ratio have been seen as early as between the eight-cell and morula stages of development [22]. While it is possible that selection of most embryos for transport was done on Day 6 after insemination exacerbated the skewed sex ratio, Block et al. [22] reported that 64% of calves born as a result of transfer of IVP embryos cultured in modified KSOM were male, even though embryos were harvested for transfer on Day 8 after insemination.

In conclusion, based on the outcome of the present study, unilateral transfer of two embryos to increase pregnancy rate is unwarranted. That fetal loss occurred sooner for heifers than cows emphasizes the importance of uterine capacity as a limiting factor for maintenance of fetal development of two conceptuses. There was also no evidence that GnRH treatment at Day 11 after anticipated ovulation improved pregnancy rate. Finally, the suitability of timed embryo transfer as a method for preparing recipients for transfer was evident by the high pregnancy and calving rates achieved with crossbred females that received a single embryo. Additional research is warranted to reduce incidence of skewed sex ratio. While sexed semen could be used to control sex ratio [48], it is likely that the underlying biological causes of altered sex ratio affect other aspects of embryo physiology also.

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References

[1] Massip A, Mermillod P, Dinnyes A. Morphology and biochemistry of in vitro produced bovine embryos: implications for their cryopreservation. Hum Reprod 1995;10:3004–11.

[2] Crosier A, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of bovine blastocysts produced in vivo or in vitro. Biol Reprod 2001;64:1375–85.

[3] Iwasaki S, Hamano S, Kuwayama M, Ushijima H, Nagaoka S, Nakahara T. Developmental changes in the incidence of chromosomal anomalies of bovine embryos fertilized in vitro. J Exp Zool 1992;261:79–85.

[4] Bertolini M, Beam SW, Shim H, Bertolini LR, Moyer AL, Famula TR, et al. Growth, development, and gene expression by in vivo- and in vitro-produced day 7 and 16 bovine embryos. Mol Reprod Dev 2002;63:318–28.

[5] Lazzari G, Wrezycki C, Herrmann D, Duchi R, Kruip T, Niemann H, et al. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol Reprod 2002;67:767–75.

[6] Lonergan P, Rizos D, Gutierrez-Adan A, Moreira PM, Pintado B, De La Fuente J, et al. Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage in vitro or in vivo. Biol Reprod 2003; 69:1424–31.

[7] Khurana NK, Niemann H. Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. Biol Reprod 2000;62:847–56.

[8] Viuff D, Greve T, Avery B, Hyttel P, Brockhoff PB, Thomsen PD. Chromosome aberrations in in vitro-produced bovine embryos at days 2–5 post-insemination. Biol Reprod 2000;63: 1143–8.

[9] Hasler JF. The current status and future of commercial embryo transfer in cattle. Anim Reprod Sci 2003;79:245–64.

[10] Rutledge JJ. Use of embryo transfer and IVF to bypass effects of heat stress. Theriogenology 2001;55:105–11.
[11] Hansen PJ, Block J. Towards an embryocentric world: the current and potential uses of embryo technologies in dairy production. Reprod Fertil Dev 2004;16:1–14.
[12] Heyman Y, Chesné P, Chupin D, Ménézo Y. Improvement of survival rate of frozen cattle blastocysts after transfer with trophoblastic vesicles. Theriogenology 1987;27:477–84.
[13] Del Campo MR, Rowe RF, French LR, Ginther OJ. Unilateral relationship of embryos and the corpus luteum in cattle. Biol Reprod 1977;16:580–5.
[14] Del Campo MR, Rowe RF, Chaichareon D, Ginther OJ. Effect of the relative locations of embryo and corpus luteum on embryo survival in cattle. Reprod Nutr Dev 1983;23:303–8.
[15] Bertolini M, Mason JB, Beam SW, Carneiro GF, Sween ML, Kominek DJ, et al. Morphology and morphometry of in vivo- and in vitro-produced bovine concepti from early pregnancy to term and association with high birth weights. Theriogenology 2002; 58:973–94.
[16] Anderson GB, Cupps PT, Drost M. Induction of twins in cattle with bilateral and unilateral embryo transfer. J Anim Sci 1979; 49:1037–42.
[17] Sreenan JM, Diskin MG. Effect of a unilateral or bilateral twin embryo distribution on twinning and embryo survival rate in the cow. J Reprod Fertil 1989;87:657–64.
[18] Reichenbach HD, Liebrich J, Berg U, Brem G. Pregnancy rates and births after unilateral or bilateral transfer of bovine embryos produced in vitro. J Reprod Fertil 1992;95:363–70.
[19] Rowson LE, Lawson RA, Moor RM. Production of twins in cattle by egg transfer. J Reprod Fertil 1971;25:261–8.
[20] Sheldon IM, Dobson H. Effects of gonadotrophin releasing hormone administered 11 days after insemination on the pregnancy rates of cattle to the first and later services. Vet Rec 1993; 133:160–3.
[21] Willard S, Gandy S, Bowers S, Graves K, Elias A, Whisnant C. The effects of GnRH administration postinsemination on serum concentrations of progesterone and pregnancy rates in dairy cattle exposed to mild summer heat stress. Theriogenology 2003;59:1799–810.
[22] Block J, Drost M, Monson RL, Rutledge JJ, Rivera RM, Paula-Lopes FF, et al. Use of insulin-like growth factor-I during embryo culture and treatment of recipients with gonadotropin-releasing hormone to increase pregnancy rates following the transfer of in vitro-produced embryos to heat-stressed, lactating cows. J Anim Sci 2003;81:1590–602.
[23] Ryan DP, Snijders S, Condon T, Grealy M, Sreenan J, O'Farrell KJ. Endocrine and ovarian responses and pregnancy rates in dairy cows following the administration of a gonadotrophin releasing hormone analog at the time of artificial insemination or at mid-cycle post insemination. Anim Reprod Sci 1994;34: 179–91.
[24] Savio JD, Boland MP, Roche JF. Development of dominant follicles and length of ovarian cycles in post-partum dairy cows. J Reprod Fertil 1990;88:581–91.
[25] Portaluppi MA, Stevenson JS. Pregnancy rates in lactating dairy cows after presynchronization of estrous cycles and variations of the Ovsynch protocol. J Dairy Sci 2005;88:914–21.
[26] Fischer-Brown A, Monson R, Parrish J, Rutledge J. Cell allocation in bovine embryos cultured in two media under two oxygen concentrations. Zygote 2002;10:341–8.
[27] Roth Z, Hansen PJ. Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. Reproduction 2005;129:235–44.
[28] Soto P, Natzke RP, Hansen PJ. Identification of possible mediators of embryonic mortality caused by mastitis: actions of lipopolysaccharide, prostaglandin $F_{2\alpha}$, and the nitric oxide generator, sodium nitroprusside dihydrate, on oocyte maturation and embryonic development in cattle. Am J Reprod Immunol 2003; 50:263–72.
[29] Echternkamp SE, Gregory KE, Dickerson GE, Cundiff LV, Koch RM, Van Vleck LD. Twinning in cattle: II. Genetic and environmental effects on ovulation rate in puberal heifers and postpartum cows and the effects of ovulation rate on embryonic survival. J Anim Sci 1990;68:1877–88.
[30] López-Gatius F, Hunter RH. Spontaneous reduction of advanced twin embryos: its occurrence and clinical relevance in dairy cattle. Theriogenology 2005;63:118–25.
[31] Ginther OJ, Knopf L, Kastelic JP. Temporal associations among ovarian events in cattle during oestrus cycles with two and three follicular waves. J Reprod Fertil 1989;87:223–30.
[32] Ko JC, Kastelic JP, Del Campo MR, Ginther OJ. Effects of a dominant follicle on ovarian follicular dynamics during the oestrous cycle in heifers. J Reprod Fertil 1991;91:511–9.
[33] Sartori R, Fricke PM, Ferreira JC, Ginther OJ, Wiltbank MC. Follicular deviation and acquisition of ovulatory capacity in bovine follicles. Biol Reprod 2001;65:1403–9.
[34] Amann RP. Weaknesses in reports of "fertility" for horses and other species. Theriogenology 2005;63:698–715.
[35] Dransfield MB, Nebel RL, Pearson RE, Warnick LD. Timing of insemination for dairy cows identified in estrus by a radiotelemetric estrus detection system. J Dairy Sci 1998;81: 1874–82.
[36] Thatcher WW, Collier RJ. Effects of climate on bovine reproduction. In: Morrow DA, editor. Current therapy in theriogenology, 2. Philadelphia: WB Saunders; 1986. p. 301–9.
[37] Ambrose JD, Drost M, Monson RL, Rutledge JJ, Leibfried-Rutledge ML, Thatcher MJ, et al. Efficacy of timed embryo transfer with fresh and frozen in vitro produced embryos to increase pregnancy rates in heat-stressed dairy cattle. J Dairy Sci 1999;82:2369–76.
[38] Al-Katanani YM, Drost M, Monson RL, Rutledge JJ, Krininger-III CE, Block J, et al. Pregnancy rates following timed embryo transfer with fresh or vitrified in vitro produced embryos in lactating dairy cows under heat stress conditions. Theriogenology 2002;58:171–82.
[39] Bo GA, Baruselli PS, Moreno D, Cutaia L, Caccia M, Tribulo R, et al. The control of follicular wave development for self-appointed embryo transfer programs in cattle. Theriogenology 2002;57:53–72.
[40] Hasler JF. In-vitro production of cattle embryos: problems with pregnancies and parturition. Hum Reprod 2000;15(Suppl. 5): 47–58.
[41] Hansen PJ, Block J. Towards an embryocentric world: the current and potential uses of embryo technologies in dairy production. Reprod Fertil Dev 2004;16:1–14.
[42] Farin CE, Farin PW, Piedrahita JA. Development of fetuses from in vitro-produced and cloned bovine embryos. J Anim Sci 2004; 82(E-Suppl.):E53–62.
[43] Kruip TAM. den Daas JHG. In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. Theriogenology 1997;47:43–52.
[44] Rerat M, Zbinden Y, Saner R, Hammon H, Blum JW. In vitro embryo production: growth performance, feed efficiency, and hematological, metabolic, and endocrine status in calves. J Dairy Sci 2005;88:2579–93.

[45] Djemali M, Berger PJ, Freeman AE. Ordered categorical sire evaluation for dystocia in Holsteins. J Dairy Sci 1987;70:2374–84.
[46] Kimura K, Spate LD, Green MP, Roberts RM. Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. Mol Reprod Dev 2005;72:201–7.
[47] Nedambale TL, Dinnyes A, Yang X, Tian XC. Bovine blastocyst development in vitro: timing, sex, and viability following vitrification. Biol Reprod 2004;71:1671–6.
[48] Wilson RD, Weigel KA, Fricke PM, Rutledge JJ, Leibfried-Rutledge ML, Matthews DL, et al. In vitro production of Holstein embryos using sex-sorted sperm and oocytes from selected cull cows. J Dairy Sci 2005;88:776–82.