# 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[1]

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**ABSTRACT:** An experiment was conducted to determine whether pregnancy rates following the transfer of in vitro-produced embryos to heat-stressed cows could be improved by 1) culturing embryos in the presence of IGF-I and 2) treating recipients with GnRH. Lactating Holstein cows (n = 260) were synchronized using a timed ovulation protocol. Embryos were produced in vitro and cultured with or without 100 ng/mL of IGF-I. On d 7 after anticipated ovulation (d 0), a single embryo was transferred to all recipients with a palpable corpus luteum (n = 210). A subset of recipients (n = 164) was injected with either GnRH or placebo on d 11. Plasma progesterone concentrations on d 0 and 7 were used to determine the synchrony of recipients. Pregnancy was diagnosed at d 53 and 81 by rectal palpation. Among all recipients, transfer of IGF-I-treated embryos increased pregnancy rate at d 53 ($P < 0.05$) and tended to increase pregnancy rate at d 81 ($P < 0.06$). Calving rate also tended to be higher for recipients that received IGF-I-treated embryos ($P < 0.07$). Among the subset of synchronized recipients (n = 190), pregnancy rate at d 53 and d 81 and calving rate were higher ($P < 0.05$) for IGF-I-treated embryos. The GnRH tended to increase pregnancy rate at d 53 for all recipients ($P < 0.08$) and the subset of synchronized recipients ($P < 0.10$). There were no effects of GnRH ($P > 0.10$) for pregnancy rate at d 81 and calving rate. The overall proportion of male calves was 64.3%. There was no effect ($P > 0.10$) of embryo treatment or GnRH on the birth weight or sex ratio of calves. Results of this experiment indicate that treatment of embryos with IGF-I can improve pregnancy and calving rates following transfer of in vitro-produced embryos. Further research is necessary to determine whether the treatment of recipients with GnRH is a practical approach to increase pregnancy rates following in vitro embryo transfer.

Key Words: Cattle, Embryo Transfer, Gonadotropin-Releasing Hormone, Heat Stress, Insulin-Like Growth Factor

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## Introduction

Problems associated with the transfer of in vitro-produced embryos exist, which limit the use of this technology. Estrus detection can be difficult in many situations, such as for cows on concrete surfaces (Britt et al., 1986) and under heat stress (Abilay et al., 1975). Estrus detection problems can be eliminated by the use of ovulation synchronization protocols to allow for timed embryo transfer without estrus detection (Ambrose et al., 1999; Al-Katanani et al., 2002; Bo et al., 2002). Other problems include reduced pregnancy rates when compared with transfer of in vivo-derived embryos (Farin and Farin, 1995; Hasler et al., 1995; Drost et al., 1999), skewed sex ratios (van Wagten-

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donk-de Leeuw et al., 1998, 2000), and an increased proportion of calves with abnormalities in growth and organ development (Farin and Farin, 1995; van Wagtendonk-de Leeuw et al., 1998, 2000).

One approach to increasing pregnancy rates is to improve culture conditions to produce embryos with higher survival following transfer to recipients. Addition of IGF-I to culture medium can increase bovine embryonic development to the morula and blastocyst stages (Palma et al., 1997; Prelle et al., 2001; Moreira et al., 2002). It has not yet been determined whether IGF-I-treated embryos are better able to survive following transfer to recipients. A second approach is to treat recipients in a way that increases the likelihood of maintaining pregnancy. The administration of GnRH 11 to 14 d following AI has been reported to improve pregnancy rates in cattle in some cases (Macmillan et al., 1986; Sheldon and Dobson, 1993; Drew and Peters, 1994), although not in all (Stevenson et al., 1993; Ryan et al., 1994; Peters et al., 2000).

Present objectives were to determine if pregnancy rates following the transfer of in vitro-produced embryos could be improved by 1) culture of embryos in the presence of IGF-I and 2) treatment of recipients with GnRH at d 11 following anticipated ovulation.

## Materials and Methods

### *Materials*

All materials were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ) unless otherwise specified. The media Sperm-Tyrode's Lactate (**TL**), IVF-TL, and HEPES-TL were purchased from Cell and Molecular Technologies Inc. (Lavallete, NJ) and used to prepare Sperm-Tyrode's Albumin Lactate Pyruvate (**TALP**), IVF-TALP, and HEPES-TALP as previously described (Parrish et al., 1986). Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Insulin-like growth factor-1 was purchased from Upstate Biotechnology (Lake Placid, NY). The $PGF_{2\alpha}$ was obtained from Pharmacia Animal Health (Kalamazoo, MI) and GnRH was Cystorelin from Rhone Merieux, (New York, NY). Lidocaine was from Bimeda (Riverside, MO). Serine protease from *Streptomyces griseus* was purchased from Sigma. Proteinase K was from Gentra (Minneapolis, MN), molecular-grade water was from Geno Technology Inc. (St. Louis, MO), allele-specific primers were purchased from Invitrogen (Grand Island, NY), and NuSieve agarose gel was obtained from BioWhittaker Molecular Applications (Rockland, MD). All other PCR reagents were purchased from Applied Biosystems (Foster City, CA).

Oocyte collection medium was tissue culture medium-199 (**TCM-199**) with Hank's salts without phenol red supplemented with 2% (vol/vol) heat-treated fetal calf serum (Gibco BRL, Grand Island, NY), 0.04 USP U of heparin/mL, 100 U/mL of penicillin-G, 0.1 mg/mL of streptomycin, and an additional 1 m*M* glutamine. Oocyte maturation medium was TCM-199 (BioWhittaker, Walkersville, MD) with Earle's salts supplemented with 10% (vol/vol) heat-treated fetal calf serum, 1 μg/mL of estradiol 17-$\beta$, 3 μg/mL of bovine LH (Sioux Biochemical, Sioux Center, IA), 3 μg/mL of bovine FSH (Sioux Biochemical), 0.2 *M* sodium pyruvate, and 50 μg/mL of gentamicin sulfate. Embryo culture medium was potassium simplex optimized medium (**KSOM**; Cell and Molecular Technologies, Lavallete, NJ), which contains 1 mg/mL of BSA. On the day of use, KSOM was modified by adding 3 mg/mL of essential fatty acid-free bovine serum albumin, 2.5 μg/mL gentamicin, essential amino acids (basal medium Eagle), and nonessential amino acids (minimal essential medium).

### *In Vitro Production of Embryos*

Procedures for oocyte collection were as previously described (Agca et al., 1998). Briefly, cumulus oocyte complexes (**COC**) were collected weekly (n = 16 replicates; 13 used for producing embryos for transfer and three additional replicates) beginning the last week of May and continuing through September 2001 at the University of Wisconsin from ovaries obtained at a local abattoir (Peck Packing Co., Milwaukee, WI). Following collection, COC were placed into 12 × 75-mm sterile polystyrene tubes containing oocyte maturation medium (approximately 50 COC per tube). The tubes were then sealed with parafilm, placed in a portable incubator (Minitube of America, Verona, WI) set at 39.0°C, and shipped overnight to Florida.

Upon arrival (approximately 24 h after collection), the COC were washed once in HEPES-TALP and fertilized with frozen-thawed semen from a single Holstein bull (American Breeders Service Global, DeForest, WI). Fertilization and embryo culture were conducted as described elsewhere (Rivera and Hansen, 2001), except that presumptive zygotes were cultured in groups of approximately 30 in 50-μL drops of modified KSOM. Approximately half the putative zygotes were cultured in KSOM containing 100 ng/mL of IGF-I for the entire culture period. For addition of IGF-I to KSOM, a vial containing 25 μg of lyophilized IGF-I was rehydrated with 100 μL of 0.1 *M* acetic acid. This solution was then stored at −20°C in 5 μL aliquots until use. At the time of use, a 5-μL aliquot of IGF-I was diluted with KSOM to 100 ng/mL. The remaining putative zygotes were used as controls and cultured in KSOM that contained the same volume of 0.1 *M* acetic acid (0.4 μL/mL). On d 7 (n = 16 replicates) and 8 (n = 11) following insemination (d 0 = day of insemination), the proportion of embryos at the blastocyst and advanced-blastocyst stage (expanded or hatched) was recorded.

Blastocyst-stage embryos were harvested on d 7 (n = 2 replicates) or 8 (n = 11) after fertilization, placed into sterile 2-mL microcentrifuge tubes containing TL-HEPES in groups of 7 to 23, and then transported

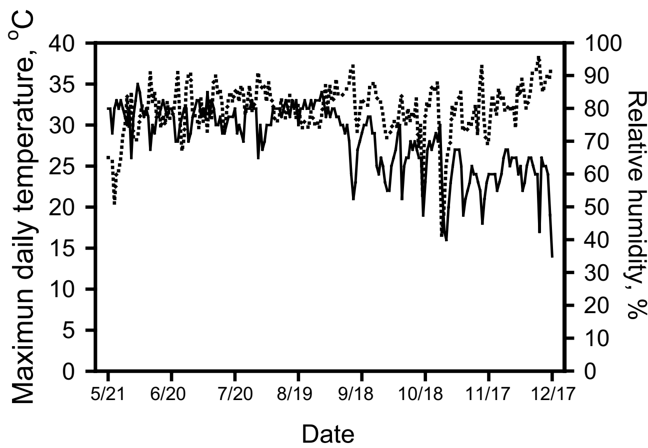


**Figure 1**. Daily maximal dry bulb temperatures (solid line) and average relative humidities (dotted line) for May 21 to December 17, 2001.

to the research site in a portable incubator set at 39°C. After transport to the research site, Grade 1 (n = 201) or 2 (n = 9) (Robertson and Nelson, 1998) blastocysts were loaded into 0.25-cc French straws in TL-HEPES (BioWhittaker, Walkersville, MD) for transfer to recipients.

### Experimental Design

The experiment was conducted from the end of May through September 2001 at the University of Florida Dairy Research Unit (Hague, Florida). The maximal daily temperature and average relative humidity for May 21 through December 17, 2001, as recorded by the Florida Automated Weather Network at Alachua, Florida, are shown in Figure 1. This time period begins 12 d before the first transfers were completed and includes the period when all d-81 pregnancy diagnoses were performed.

A total of 210 primiparous and multiparous lactating Holstein cows housed in freestall barns equipped with fans and sprinkler systems were used as recipients. All recipients received bovine somatotropin as directed by the manufacturer (Monsanto, Chesterfield, MO) and were fed a total mixed ration and milked three times per day. Overall, 13 replicates were completed with between 6 and 24 recipients per replicate.

To synchronize cows for timed embryo transfer, all first-service cows (39 to 45 d in milk) were presynchronized with two injections of 25 mg of $PGF_{2\alpha}$, i.m. 14 d apart, followed 12 d later by the initiation of a timed ovulation protocol (100 μg of GnRH, i.m. on d −10; 25 mg of $PGF_{2\alpha}$, i.m. on d −3; 100 μg of GnRH i.m. on d −1; d 0 = day of anticipated ovulation). All cows that were diagnosed not pregnant during the course of the experiment, as well as all other return-to-service cows, were also synchronized for timed embryo transfer using the timed ovulation protocol without presynchronization. To decrease the interval between pregnancy diagnosis and rebreeding, all cows that were due to be diagnosed for pregnancy were given the first GnRH injection of the timed ovulation protocol at 1 wk prior to the scheduled day of pregnancy diagnosis. In this way, all cows diagnosed not pregnant were given the $PGF_{2\alpha}$ injection of the timed ovulation protocol on the day of pregnancy diagnosis and the second GnRH injection 2 d later.

On d 7 after anticipated ovulation, all cows were palpated per rectum for the presence of a corpus luteum and BCS were recorded (Edmonson et al., 1989). Cows that had a palpable corpus luteum received an epidural block of 5 mL of lidocaine (2%), and a single embryo was then transferred to the uterine horn ipsilateral to the ovary containing the corpus luteum. For the last 10 replicates (n = 164 recipients), 100 μg of GnRH (i.m.) or placebo (9 mg/mL of benzyl alcohol and 7.47 mg/mL of sodium chloride in water) was administered randomly to recipients on d 11 after anticipated ovulation. Pregnancy was diagnosed by rectal palpation at d 53 and 81. The birth weight, sex, and any abnormalities of calves were recorded at birth.

### Blood Sample Collection and Progesterone Radioimmunoassay

Progesterone concentrations were measured to determine the proportion of cows in which ovulatory cycles were successfully synchronized. Blood samples were collected on d 0 and 7 by coccygeal venipuncture into evacuated heparinized tubes (Becton Dickinson, Franklin Lakes, NJ). Following collection, blood samples were placed in an ice chest until further processing at the laboratory (approximately 2 to 4 h). Blood samples were centrifuged at 2,000 × *g* for 20 min at 4°C. Plasma was separated and stored at −20°C until assayed for progesterone. Plasma progesterone concentration was determined using the Coat-a-Count progesterone RIA kit (Diagnostic Products Corp., Los Angeles, CA). The sensitivity of the assay was 0.1 ng/mL and the intrassay and interassay CV were 8.3 and 10.0%, respectively.

### Embryo Sexing

To determine the sex ratio obtained using the in vitro production system, 105 embryos were produced in two replicates and sexed by amplification of the *zfx* and *zfy* genes using the PCR. Procedures for embryo production were generally similar to those described above except that oocyte collection and maturation was conducted as described elsewhere (Rivera and Hansen, 2001). For the first replicate, only blastocyst stage embryos were harvested on d 8 after fertilization. On the second replicate, all embryos >4 cells were harvested for sex determination. Embryos were removed from culture and washed in PBS containing 0.3% (wt/vol) polyvinyl pyrrolidone (**PVP**). After

washing several times, each embryo was placed in a small volume (approximately 25 μL) of PBS-PVP containing 0.5% (wt/vol) serine protease at 39°C for approximately 1 min to remove the zona pellucida. Zona pellucida-free embryos were washed individually in PBS-PVP several times and then placed individually into 250-μL PCR tubes containing 5 μL of lysis buffer (2% [vol/vol] 2-mercaptoethanol, 1.0% [vol/vol] SDS, 10 m*M* EDTA, 10 m*M* Tris, pH 8.3, 222 μg/mL proteinase K) and shipped overnight to Vicam (Watertown, MA). Embryo lysis was carried out at 55°C for 1 h. Proteinase K was then inactivated at 98°C for 10 min. After lysis, 95 μL of molecular grade water was added to each embryo lysis tube to dilute the samples.

A 5-μL sample from each lysis tube was used for PCR amplification. The PCR protocol utilized was a modification of the PCR protocol described by Kirkpatrick and Monson (1993). Briefly, the first round of PCR was completed using primers complementary to both *zfx* and *zfy* genes. The nested PCR was conducted to specifically amplify the *zfx* or *zfy* gene and to generate 247-bp (*zfx*) and 167-bp (*zfy*) products. The PCR cocktail consisted of 1× GeneAmp PCR Gold buffer (15 m*M* Tris-HCl, pH 8.0, 50 m*M* KCl), 2.5 m*M* $MgCl_2$, 45 μ*M* of each deoxynucleotidetriphosphate, 250 n*M* of each primer, and 1 unit of AmpliTaq Gold DNA polymerase per 50-μL reaction. The first round of PCR was done by hot-start at 94°C for 10 min. Five triphasic cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min were performed followed by 25 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s with final extension at 72°C for 10 min. A 2-μL sample of primary PCR product was used for nested PCR. The *zfx* and *zfy* genes were amplified in separate tubes using the cycling protocol cited above. Following amplification, 7 μL of PCR product was loaded onto a 1.5% (wt/vol) NuSieve agarose gel containing ethidium bromide (0.5 μg/mL) and resolved in Tris-acetate EDTA buffer by electrophoresis for 45 min at 82 V. The gel was visualized using a UV transilluminator mounted with a camera.

### *Statistical Analysis*

The proportion of oocytes that cleaved, the proportion of embryos that developed to the blastocyst stage at d 7 and 8 after fertilization, and the proportion of embryos that developed to the advanced blastocyst stages at d 7 and 8 was calculated for each replicate. Treatment effects were analyzed using least squares ANOVA with the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The model included the main effects of week of oocyte collection and IGF-I. Week of oocyte collection was considered random and IGF-I treatment was considered fixed. Percentage data were transformed by arcsin transformation before analysis. Probability values are reported from analysis of transformed data, whereas least squares means ± SEM are derived from untransformed data.

For analysis of cow data, animals were classified several ways. In addition to the effect of embryo treatment (IGF-I vs. control) and GnRH, classifications were based on BCS at the time of transfer (cows with a BCS ≤2.5 and those with a BCS >2.5), days in milk (cows ≤150 d and cows >150 d in milk), projected 305-d milk yield on the day of transfer (cows projected to yield ≤9,000 kg of milk and those projected to yield >9,000 kg of milk), and plasma progesterone concentration on the day of transfer (cows with progesterone ≤2.5 ng/mL and those with progesterone >2.5 ng/mL). For statistical analysis, each transfer was considered to be an independent observation, although some recipients were used more than once during the course of the experiment.

Synchronization, pregnancy, and calving data were analyzed by logistical regression using the LOGISTIC procedure of SAS. Probability values from PROC LOGISTIC were obtained using WALD statistics derived from type-III analyses. Synchronization, pregnancy, and calving data were also analyzed by ANOVA using the GLM procedure of SAS. Initial models included all main effects and two-way interactions. Data were subsequently reanalyzed after removing nonsignificant effects from the model. Because there was no interaction between embryo treatment and GnRH treatment among the subset of 164 recipients, the effect of embryo treatment on the dependent variables outlined below was analyzed using the entire data set (n = 210). The effect of GnRH treatment was analyzed both among all recipients (three treatments—no GnRH, placebo, and GnRH), as well as among the subset of cows (n = 164) that were given either GnRH or placebo on d 11 after anticipated ovulation. Probability values for the effect of GnRH were derived from the analysis of the subset of recipients. The effects of all other independent variables were analyzed using the whole data set.

To determine the response to the synchronization protocols, cows were classified based on plasma progesterone concentrations on d 0 and 7. Cows that had ≤1.0 ng/mL plasma progesterone on the day of anticipated ovulation and >1.0 ng/mL plasma progesterone on the day of transfer were considered to be synchronized at the time of transfer. Five cows were excluded from the analysis because one or both blood samples were missing. Two recipients with one missing sample at d 0 were considered synchronized based on observed estrus on d 0 and plasma progesterone concentration >1.0 ng/mL on the day of transfer. For statistical analysis of synchronization rate, models included effects of days in milk class, milk yield class, month of transfer, and two-way interactions. Body condition scores were not recorded for cows that did not receive an embryo; therefore, BCS and BCS class were not included in the analysis of synchronization rate.

Based on palpation per rectum on the day of transfer, 210 recipients were selected to receive embryos. Pregnancy rate at d 53 and 81 and calving rate were analyzed among all 210 recipients, as well as among a subset of the 210 recipients (n = 190) that were considered synchronized based on plasma progesterone concentrations. Calving rate among recipients was described two ways: 1) as the proportion of recipients that gave birth to a calf, dead or alive (termed calving rate—all calves) and 2) as the proportion of recipients that gave birth to a calf that survived at least 24 h (termed calving rate—live calves). Models included effects for month of transfer, embryo treatment, GnRH, BCS as a class variable or continuous variable, plasma progesterone concentration on the day of transfer as a class variable or continuous variable, days in milk class, milk yield class, and two-way interactions.

Pregnancy loss was calculated as the loss between d 54 and 81, d 81 and calving, and between d 54 and calving. For this purpose, birth of a dead calf was not considered a pregnancy loss. The rate of perinatal mortality, defined as the birth of a dead calf or calf death within 24 h of birth, was also calculated. Treatment effects on pregnancy loss and perinatal mortality were analyzed using the same variables used to describe pregnancy and calving rate above. Pregnancy loss data were analyzed by the LOGISTIC procedure or, when some subgroups experienced no losses, by using the $\chi^2$ option of PROC FREQ of SAS. The $\chi^2$ analysis was performed because logistic regression analysis is not valid in cases where one or more treatment combinations have a frequency of zero.

Data for calf birth weight were analyzed among all calves that were born and also among all live calves born using the GLM procedure of SAS. Models included month of transfer, embryo treatment, GnRH, sex, BCS as a class variable or continuous variable, plasma progesterone concentration on the day of transfer as a class variable or continuous variable, days in milk class, milk yield class, and all two-way interactions.

Data for sex ratio were analyzed as described for pregnancy and calving data using both PROC LOGISTIC and PROC GLM. Initial models were similar to those described for analysis of calf birth weight except calf sex was eliminated from the model as an independent variable. Parturition was induced in 14 out of 28 recipients at 263 to 279 d of gestation to simplify periparturient management. Thus, data for gestation length, as well as the effects of gestation length on calf birth weight and sex ratio, were not analyzed statistically.

All levels of statistical significance obtained by logistic regression were similar to those obtained from least squares ANOVA and probability values reported herein were obtained from logistical regression except for data concerning embryo development and calf birth weight (PROC GLM) and certain of the data on pregnancy loss ($\chi^2$ analysis using PROC FREQ).

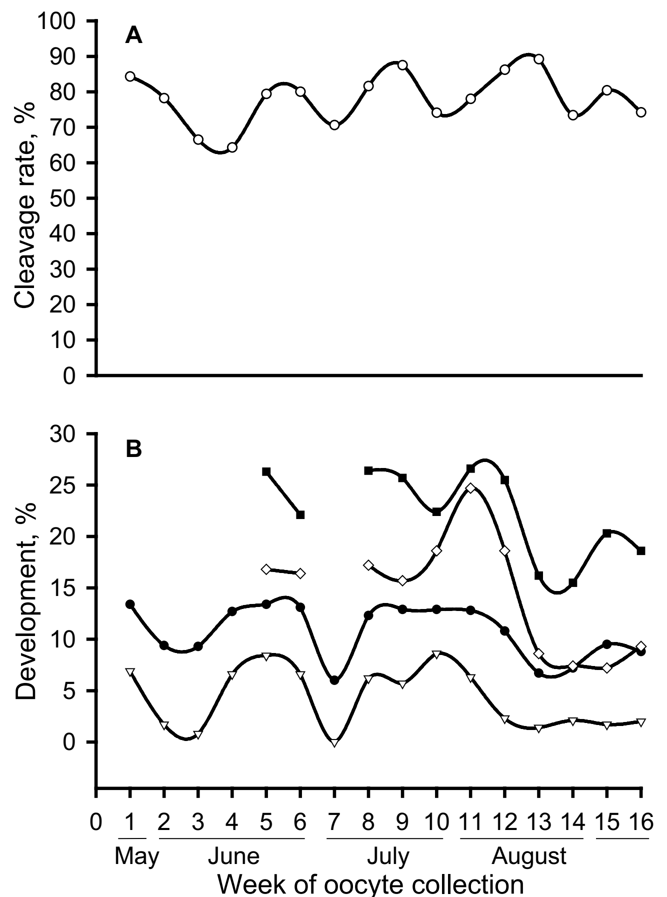


**Figure 2**. Effect of week of oocyte collection on cleavage rate and embryo development. Panel A represents the proportion of oocytes that cleaved. Panel B depicts the proportion of oocytes that developed to the blastocyst stage on d 7 (open triangle) and 8 (open diamond), as well as the proportion of oocytes that developed to advanced blastocyst stages on d 7 (closed circle) and 8 (closed square). Note that the data on d 8 was not collected for the first 4 wk of the experiment and for 1 wk in July. Data represent least squares means. The SEM was 1.8 for cleavage rate, 1.6 for percentage blastocysts at d 7, 1.5 for percentage of advanced blastocysts at d 7, 1.6 for percentage of blastocysts at d 8, and 1.3 for percentage of advanced blastocysts at d 8.

## Results

### *Embryo Development*

A total of 3,873 oocytes were collected and fertilized over 16 replicates between May and September 2001. As shown in Figure 2A, the proportion of oocytes that cleaved was affected by the week of oocyte collection ($P < 0.0001$). Furthermore, week of oocyte collection affected the proportion of oocytes that developed to the blastocyst stage on d 7 ($P < 0.05$) and 8 ($P < 0.01$)

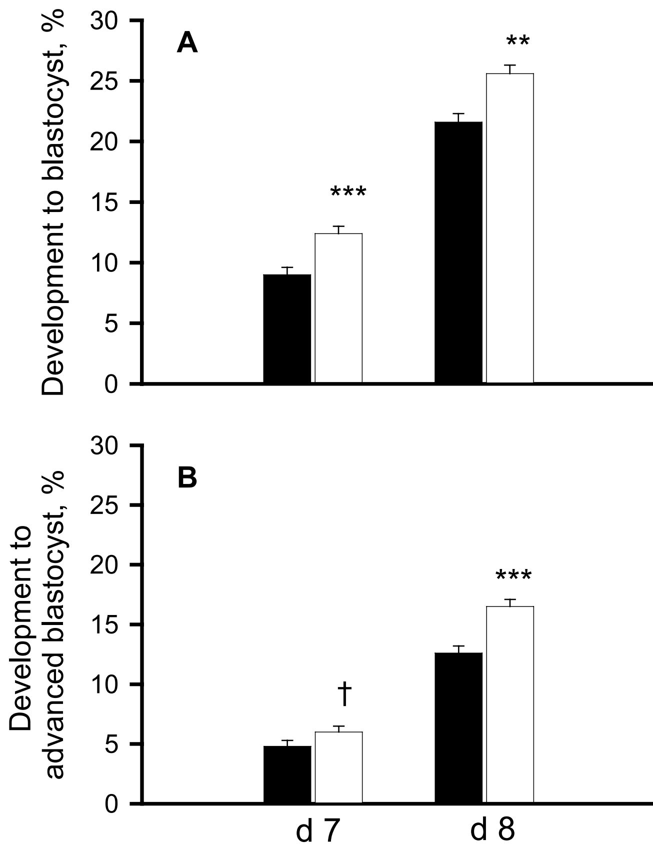


**Figure 3**. Effect of IGF-I on embryo development. Panel A represents the proportion of control (filled bar) and IGF-I (open bar) oocytes that developed to the blastocyst stage on d 7 and 8 after fertilization. Panel B depicts the proportion of control (filled bar) and IGF-I (open bar) oocytes that developed to advanced blastocyst stages (expanded and hatched) on d 7 and d 8 after fertilization. Data represent least-squares means ± SEM. †$P < 0.07$, **$P < 0.01$, ***$P < 0.001$.

following insemination, as well as the proportion of oocytes that developed to advanced blastocyst stages on d 7 ($P < 0.01$) and 8 ($P < 0.0001$) (Figure 2B). In all cases, IVF performance tended to be lower at wk 7 and the last weeks (wk 13 to 16).

Addition of IGF-I to culture medium did not affect the proportion of oocytes that cleaved, but did increase the proportion of oocytes that developed to the blastocyst stage on both d 7 ($P < 0.001$) and 8 ($P < 0.01$) following fertilization (Figure 3A). Moreover, IGF-I tended to increase the proportion of oocytes that reached advanced stages of blastocyst development on d 7 ($P < 0.07$) and increased the proportion of oocytes that developed into advanced blastocysts on d 8 ($P < 0.001$) after insemination (Figure 3B).

### *Synchronization Rate*

A total of 260 cows were subjected to synchronization for timed embryo transfer. Plasma progesterone concentrations on d 0 and 7 were analyzed for 255 cows. Overall, 86.3% (220/255) of the cows subjected to the timed ovulation protocol were considered to have successfully responded based on plasma progesterone concentrations on the day of anticipated ovulation and the day of transfer. Synchronization rate was not affected by milk yield class, days in milk class, or month of transfer.

A total of 210 recipients were selected as recipients based on the presence of a corpus luteum diagnosed via palpation per rectum. Of these 210 recipients, 190 (90.5%) were considered to be synchronized based on plasma progesterone concentrations on d 0 and 7.

### *Effects of Insulin-Like Growth Factor-1 on Pregnancy and Calving Rates*

Among all recipients, those receiving an embryo cultured in the presence of IGF-I had higher pregnancy rate at d 53 ($P < 0.05$) than cows receiving control embryos (Figure 4A). There was also a tendency ($P < 0.06$) for pregnancy rate at d 81 to be higher for cows receiving IGF-I-treated embryos. Moreover, transfer of IGF-I-treated embryos tended to increase the proportion of cows that carried a pregnancy to term ($P < 0.07$), as well as the proportion of recipients giving birth to a live calf ($P < 0.06$, Figure 4A). Among the subset of recipients that were classified as synchronized at the time of transfer based on plasma progesterone concentrations, cows receiving an IGF-I-treated embryo had higher pregnancy rates at d 53 ($P < 0.01$), 81 ($P < 0.05$), and term (all calves and live calves, $P < 0.05$) (Figure 4B).

### *Effects of Gonadotropin-Releasing Hormone on Pregnancy and Calving Rates*

Although pregnancy rates at d 53 and 81 and calving rate (all calves and live calves) among all recipients were higher for recipients that received GnRH on d 11 (Figure 5A); differences ($P < 0.08$) were observed only at d 53. Similar results were obtained among recipients that were considered synchronized at the time of transfer (Figure 5B).

### *Effects of Month of Transfer and Body Condition Score on Pregnancy and Calving Rates*

Month of transfer affected pregnancy rate at d 53 among all recipients and synchronized recipients ($P < 0.05$) where pregnancy rate declined between June and September. For all recipients, pregnancy rate at d 53 was higher for recipients that received embryos in June and July than for those that received embryos in September ($P < 0.01$ and $P < 0.05$, respectively). Pregnancy rate at d 53 for June recipients was also higher than for August recipients ($P < 0.05$). July recipients tended to have higher pregnancy rates at d 53 than August recipients ($P < 0.06$). Pregnancy rate at d 53 did not differ between recipients that received

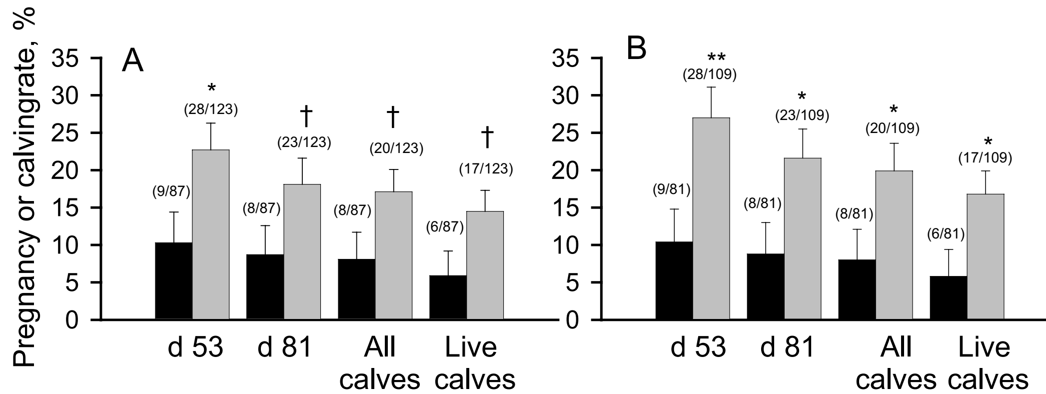


**Figure 4**. Pregnancy (d 53 and 81) and calving rates following the transfer of control (filled bar) or IGF-I-treated (shaded bar) embryos. Panel A represents the pregnancy and calving rates obtained among all recipients. Panel B represents pregnancy and calving rates among synchronized recipients. Graphed data represent least squares means ± SEM, whereas the numbers in parentheses represent the number of animals pregnant (or calving) divided by all animals. †$P < 0.06$ (d 81 and live calves) or $P < 0.07$ (all calves), *$P < 0.05$, **$P < 0.01$.

embryos in August and September (Table 1). Among the subset of synchronized recipients, pregnancy rate at d 53 was higher for June recipients than for recipients that received embryos in July ($P < 0.05$), August ($P < 0.01$), or September ($P < 0.01$). Pregnancy rates at d 53 among synchronized July recipients tended to be higher than August recipients ($P < 0.08$) and were higher ($P < 0.05$) than pregnancy rates at d 53 among synchronized September recipients. Pregnancy rates at d 53 for synchronized August and September recipients did not differ (Table 1). Whereas month of transfer affected pregnancy rate at d 53, there was no effect of month of transfer on pregnancy rate at d 81 or calving rate (total calves or live calves) for either all recipients or the subset of synchronized recipients (Table 1).

Body condition score class tended to affect pregnancy rate among all recipients and synchronized recipients at d 53 ($P < 0.07$ and $P < 0.10$, respectively) and 81 ($P < 0.07$ and $P < 0.08$), and calving rate (all calves only, $P < 0.06$ and $P < 0.08$). Recipients with BCS >2.5 at the time of transfer had numerically higher pregnancy rates at all three time points (Table 1). The calving rate (for live calves) was numerically higher for cows with BCS >2.5 at the time of transfer among all recipients and synchronized recipients, but probability values for these differences were greater than 0.10 (Table 1). Body condition score was a significant covariate of pregnancy rate among all recipients and synchronized recipients at d 53 ($P < 0.01$ and $P < 0.01$, respectively), d 81 ($P < 0.01$ and $P < 0.01$, respectively), and at term ($P < 0.05$ and $P < 0.01$ for

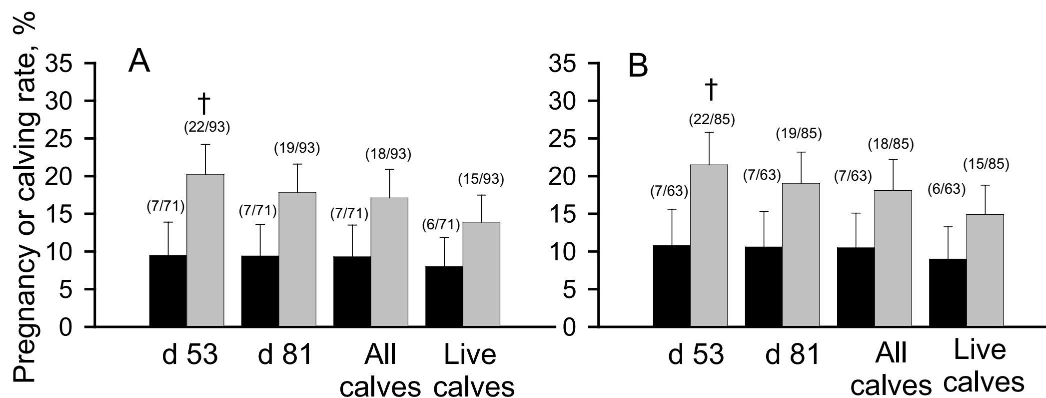


**Figure 5**. Effect of GnRH adminstration (shaded bar) vs. placebo (filled bar) at d 11 following anticipated ovulation on pregnancy (d 53 and 81) and calving rates. Panel A represents the pregnancy and calving rates obtained among all recipients. Panel B depicts the pregnancy and calving rates among synchronized recipients. Graphed data represent least-squares means ± SEM, whereas the numbers in parentheses represent the number of animals pregnant (or calving) divided by all animals. †$P < 0.08$ (panel A) or $P < 0.10$ (panel B).

**Table 1**. Effect of month of transfer, body condition score class, and plasma progesterone class on the day of transfer on pregnancy and calving rate[a]

| | Pregnancy rate, % | | | | Calving rate, % | | | |
|---|---|---|---|---|---|---|---|---|
| | d 53 | | d 81 | | All calves | | Live calves | |
| | All recipients | Synch. recipients | All recipients | Synch. recipients | All recipients | Synch. recipients | All recipients | Synch. recipients |
| Month | | | | | | | | |
| June | 38 ± 10% (6/23)[a] | 44 ± 11% (6/20)[a] | 30 ± 9% (4/23) | 33 ± 10% (4/20) | 14 ± 7% (3/23) | 17 ± 8% (3/20) | 9 ± 7% (2/23) | 12 ± 7% (2/20) |
| July | 20 ± 4% (19/84)[a,b] | 20 ± 4% (19/79)[b] | 15 ± 4% (15/84) | 16 ± 4% (15/79) | 15 ± 4% (13/84) | 15 ± 4% (13/79) | 11 ± 3% (10/84) | 12 ± 4% (10/79) |
| August | 6 ± 6% (7/57)[b,c] | 7 ± 6% (7/53)[b,c] | 6 ± 5% (7/57) | 7 ± 6% (7/53) | 12 ± 5% (7/57) | 13 ± 5% (7/53) | 10 ± 4% (6/57) | 11 ± 5% (6/53) |
| September | 3 ± 6% (5/46)[c] | 4 ± 7% (5/38)[c] | 3 ± 6% (5/46) | 5 ± 7% (5/38) | 9 ± 5% (5/46) | 11 ± 6% (5/38) | 9 ± 5% (5/46) | 11 ± 5% (5/38) |
| Body condition[b] | | | | | | | | |
| <2.5 | 10 ± 4 (9/78) | 12 ± 5% (9/68) | 8 ± 4% (7/78) | 9 ± 5% (7/68) | 7 ± 4% (6/78) | 8 ± 4% (6/68) | 6 ± 4% (5/78) | 7 ± 4% (5/68) |
| >2.5 | 20 ± 4% (28/132) | 22 ± 4% (28/122) | 17 ± 3% (24/132) | 19 ± 4% (24/122) | 16 ± 3% (22/132) | 18 ± 3% (22/122) | 13 ± 3% (18/132) | 14 ± 7% (18/122) |
| | ($P < 0.07$) | ($P < 0.10$) | ($P < 0.07$) | ($P < 0.08$) | ($P < 0.06$) | ($P < 0.08$) | | |
| Plasma[et progesterone (d 7) | | | | | | | | |
| <2.5 ng/mL | 10 ± 6% (4/41) | 14 ± 7% (4/30) | 9 ± 6% (4/41) | 13 ± 7% (4/30) | 10 ± 5% (4/41) | 13 ± 7% (4/30) | 7 ± 5% (3/41) | 9 ± 6% (3/30) |
| >2.5 ng/mL | 18 ± 3% (33/169) | 20 ± 3% (33/160) | 15 ± 3% (27/169) | 16 ± 3% (27/160) | 13 ± 3% (24/169) | 14 ± 3% (24/160) | 11 ± 3% (20/169) | 12 ± 3% (20/160) |

[a]Data outside parentheses represent least-squares means ± SEM, whereas data inside parentheses represent the actual proportions. Values in the same column with different superscipts differ, $P < 0.05$.
[b]Scale of 1 to 5.

all calves and $P < 0.05$ and $P < 0.01$ for live calves, respectively).

### *Relationships of Plasma Progesterone Concentrations with Pregnancy and Calving Rates*

There was no significant relationship between plasma progesterone concentration at the time of transfer and pregnancy rate or calving rate (Table 1). This was true whether plasma progesterone concentration was used as a covariate or whether progesterone class on the day of transfer (≤2.5 ng/mL vs. >2.5 ng/mL) was used in the analysis and whether data were analyzed from all recipients or were restricted to synchronized recipients (Table 1). Furthermore, there was no difference in mean plasma progesterone concentration on the day of transfer between recipients that ultimately maintained pregnancy and those that did not (3.75 ± 0.27 ng/mL vs. 3.83 ± 0.13 ng/mL for all recipients and 3.82 ± 0.31 ng/mL vs. 3.87 ± 0.14 ng/mL for synchronized recipients, respectively).

### *Pregnancy Loss and Perinatal Mortality*

Overall, a total of nine pregnant recipients lost their pregnancy between d 53 and calving (9/37 = 24.3%). Between d 53 and 81, pregnancy loss was 16.2% (6/37), whereas pregnancy loss between d 81 and calving was 9.7% (3/31). Month of transfer tended to affect pregnancy loss between d 53 and calving ($P < 0.09$). Among recipients receiving embryos in the months of August and September, there was no pregnancy loss between d 53 and calving (0/7 in August and 0/5 in September), whereas there was a 50% (3/6) loss among June recipients and a 32% (6/19) loss among July recipients. There was no effect of month of transfer on pregnancy loss between d 53 and 81 or between d 81 and calving. Embryo treatment and GnRH did not alter pregnancy loss at any time point. Furthermore, there was no effect of BCS at the time of transfer, BCS class at the time of transfer, plasma progesterone concentration, or plasma progesterone class on the day of transfer on pregnancy loss between d 53 and 81, pregnancy loss between d 81 and calving, or pregnancy loss between d 53 and calving.

Of the 28 calves born in the study, 5 (17.9%) calves were born dead or died within 24 h of birth. This included one calf removed at parturition by fetotomy and 1 case of hydrocephalus. There was no significant effect of month of transfer, embryo treatment, GnRH treatment, sex, birth weight, gestation length, or any other main effect on the proportion of calves born that remained alive for at least 24 h.

### *Calf Birth Weight*

The overall mean birth weight was 43.7 kg. Among the 28 calves born, there were 13 Holstein crossbred calves and 15 Holstein calves. The mean birth weight for the crossbred calves and for the Holstein calves

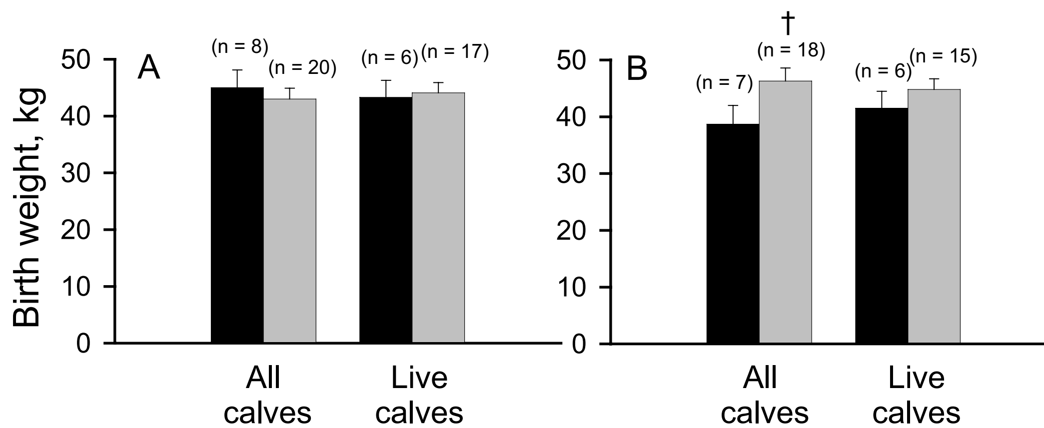


**Figure 6**. Effect of embryo treatment and GnRH on calf birth weights. Panel A represents the birth weight of all calves and live calves born from control (filled bar) and IGF-I-treated (shaded bar) embryos. Panel B represents the effect of treatment with placebo (filled bar) or GnRH (shaded bar) at d 11 following anticipated ovulation on calf birth weights among all calves and live calves. Data represent least squares means ± SEM. †$P < 0.06$.

was 45.0 and 42.5 kg, respectively. Three calves had very large birth weights (54.5 kg or more); two weighed 54.5 kg (1 born alive) and 1 weighed 56.8 kg (born alive). Only one caesarian section was performed with the resulting live calf weighing 46.4 kg.

There was no effect of IGF-I treatment of embryos on calf birth weight whether calculated for all calves born or for live calves only (Figure 6A). Treatment of recipients with GnRH tended to increase calf birth weight among all calves ($P < 0.06$) born, but there was no significant effect on birth weight among live calves (Figure 6B). There was also no significant effect of sex on calf birth weight among all calves born (42.3.0 ± 2.3 kg vs. 44.9 ± 3.1 kg for males and females, respectively) or live calves (43.3 ± 2.3 kg vs. 45.4 ± 3.6 kg for males and females, respectively). Month of transfer, BCS, BCS class, plasma progesterone concentration on the day of transfer and plasma progesterone class on the day of transfer did not significantly affect calf birth weight.

### *Sex Ratio*

Although not significantly different from an expected 50:50 male:female ratio, there was a higher proportion of male calves born (18/28 = 64.3% for all calves and 16/23 = 69.6% for live calves). There was no significant effect of IGF-I (Figure 7A) or GnRH (Figure 7B), month of transfer, BCS, BCS class, plasma progesterone concentration on the day of transfer, or plasma progesterone class on the day of transfer on calf sex ratio for all calves born or for live calves only.

The sex ratio of embryos produced in our laboratory and sexed using nested PCR was similar to the sex ratio obtained among the calves produced in the em-

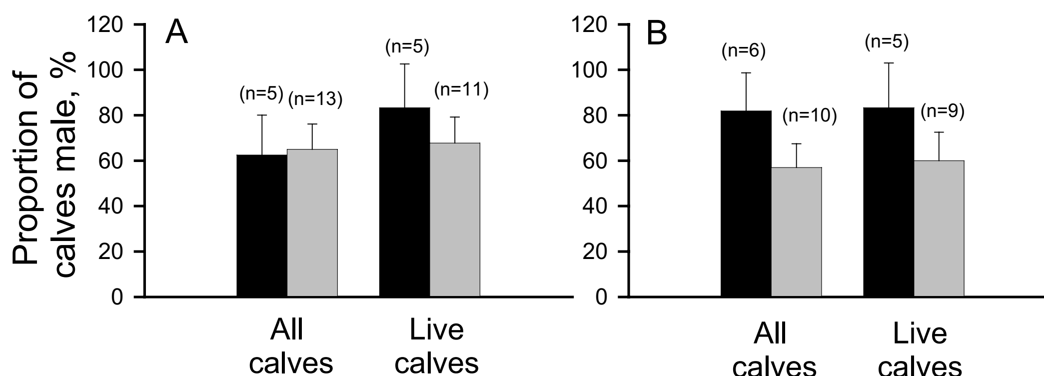


**Figure 7**. Effect of embryo treatment and GnRH on calf sex ratio. Panel A represents the sex ratio among all calves and live calves produced from control (filled bar) and IGF-I-treated (shaded bar) embryos. Panel B represents the effect of administration of placebo (filled bar) or GnRH (shaded bar) to recipients at d 11 after anticipated ovulation on sex ratio among all calves and live calves. Data represent least squares means ± SEM.

bryo transfer study. Of the 59 embryos that were at the compact morula stage or greater on d 8 after fertilization, 41 (69.5%) were males and 18 (30.5%) were females. Interestingly, the sex ratio for embryos past the 8 cell stage but not the morula stage on d 8 following fertilization also favored males (74.0% [34/46] males and 26.0% [12/46] females.

## Discussion

The goal of this study was to determine whether pregnancy rates and calving rates following TET with in vitro-produced embryos can be improved by treating embryos in a manner to improve their survival following transfer (i.e., by adding IGF-I to culture medium) as well as by treating recipients hormonally to enhance the probability that a transferred embryo would establish a pregnancy (i.e., by injection of GnRH at d 11 after presumed ovulation). Results indicate that pregnancy rates can indeed be increased by IGF-I treatment of embryos. Although not significant, the tendency for GnRH to increase pregnancy rates makes further research into this area worthwhile. In addition, the present results also point out some limitations to optimal use of embryo transfer of in vitro-produced embryos including high fetal loss and altered sex ratio.

Week of oocyte collection in the present study affected the proportion of oocytes that developed into blastocysts and advanced blastocysts on both d 7 and 8 following insemination. The percentage of oocytes that developed into blastocysts and advanced blastocysts on d 7 and 8 after insemination declined during the last 4 wk of the experiment (August and September). It has been reported that blastocyst yields from oocytes collected in Wisconsin decline in mid- to late summer (Rutledge et. al, 1999). Thus, the decline in embryo production during the last few weeks in the present study may be due to donor cows in Wisconsin experiencing heat stress.

As has been previously reported (Palma et al., 1997; Prelle et al., 2001; Moreira et al., 2002), addition of IGF-I to culture medium increased embryonic development. In particular, IGF-I increased the proportion of oocytes that developed to the blastocyst and advanced-blastocyst stages at d 7 and 8 after fertilization. Furthermore, the present study indicated that embryos cultured in the presence of IGF-I are also better able to survive following transfer to recipients. The observation that recipients receiving an embryo cultured in the presence of IGF-I had increased pregnancy rates and calving rates is, to our knowledge, the first study to demonstrate that growth-factor treatment of embryos can improve embryo survivability following transfer.

The mechanism by which IGF-I increases embryonic survivability following transfer is not known. However, addition of IGF-I to culture medium has been reported to increase blastocyst cell number (Makarevich and Markkula, 2002; Moreira et al., 2002) and decrease the proportion of blastomeres that are apoptotic (Makarevich and Markkula, 2002). Thus, IGF-I-treated embryos may be more likely to establish pregnancy after transfer because they have a greater number of cells and a higher proportion of viable cells. Addition of IGF-I to embryo culture may also improve embryo survival by improving embryo metabolism during the preimplantation period. It has been reported previously that IGF-I can increase glucose uptake by murine blastocysts (Pantaleon and Kaye, 1996). Furthermore, IGF-I may affect glucose transporter 8 translocation. It has been reported that downregulation of the IGF-I receptor affects glucose transporter 8 translocation in murine blastocysts, and that glucose transporter 8 expression is important for embryo survival following transfer in mice (Pinto et al., 2002).

Although differences did not reach the level of statistical significance, pregnancy, and calving rates were numerically higher (refer to Figure 4) for recipients treated with GnRH at d 11 after anticipated ovulation than for recipients treated with placebo. To our knowledge, this is the first study in which a GnRH analog has been administered during the mid-luteal phase following embryo transfer. Many studies have been performed to evaluate effect of GnRH treatment 11 to 14 d after AI on subsequent pregnancy rates, and outcomes have been variable. Some have reported increased fertility following GnRH treatment (Macmillan et al., 1986; Sheldon and Dobson, 1993; Drew and Peters, 1994), whereas others have reported no effect (Stevenson et al., 1993; Ryan et al., 1994). Administration of GnRH 11 to 14 d after estrus has been reported to increase plasma and serum concentrations of both LH and progesterone (Macmillan et al., 1985; Rettmer et al., 1992; Stevenson et al., 1993). Injection of GnRH during the mid-luteal phase increased blood concentrations of progesterone in dairy heifers (Rettmer et al., 1992) and lactating dairy cows (Macmillan et al., 1985; Stevenson et al., 1993). High concentrations of progesterone following embryo transfer may improve embryo survival by increasing conceptus development (Garrett et al., 1992) and interferon-$\tau$ production (Mann and Lamming, 2001) and by inhibiting prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion (Bogacki et al., 2002). The fact that GnRH tended to increase pregnancy rate following transfer indicates that additional studies to evaluate the effectiveness of this treatment are warranted.

Overall pregnancy loss between d 53 and calving in the present study was 24%. In other studies, pregnancy loss between d 50 to 60 and term following the transfer of a single in vitro-produced embryo has ranged from 4–13% (Hasler, 2000; van Wagtendonk-de Leeuw et al., 2000; Heyman et al., 2002). Similar to results in other embryo transfer studies (van Wagtendonk-de Leeuw et al., 2000; Heyman et al., 2002), there was more pregnancy loss between d 53 and 81

than thereafter. There are at least two possible reasons for the high pregnancy loss in the present study. One is the lactational state of the recipients—lactating cows in the present experiment vs. Holstein heifers (Hasler et al., 2000) or beef heifers (Heyman et al., 2002). Lactation is associated with altered endocrine status, including reduced progesterone concentrations (de le Sota et al., 1993; Chagas e Silva et al., 2002), and such changes could conceivably affect fetal survival. Another possible explanation for the high pregnancy loss in the present experiment may be the heat stress that recipients experienced. Whereas month of transfer did not significantly affect pregnancy loss, there was a nonsignificant trend for greater pregnancy loss among recipients that received embryos in the early part of the summer. In fact, all pregnancy losses occurred among recipients that received embryos in the months of June and July. Of the nine pregnancies that were lost among June and July recipients, six (67%) were lost sometime between d 53 and 81 (i.e., during the months of August, September, and early October). Although it is known that heat stress can compromise placental function and growth of the fetus (Collier et al., 1982; Wolfenson et al., 1988), little is known about whether heat stress causes fetal loss.

Perinatal mortality in the current study was 17.9% (5/28). This value is comparable to that reported previously for in vitro-produced embryos in one study (15.6%; Hasler et al., 1995) but is much higher than the 3.1 to 10.0% perinatal mortality reported by others (Mayne and McEvoy, 1993; van Wagtendonk-de Leeuw et al., 1998; 2000). There was no significant effect of month of transfer, embryo treatment, or GnRH treatment on calf loss. Furthermore, perinatal mortality was not altered by calf sex or calf birth weight.

The mean birth weight was 43.7 kg for all calves and 43.9 kg for all live calves. For Holsteins, the mean birth weight was 42.5 kg, a value comparable to the mean birth weight of 41.4 kg obtained from a large data set of Holstein cows (Kertz et al., 1997). Although the addition of IGF-I to embryo culture medium has been reported to increase blastocyst cell number (Moreira et al., 2002), there was no effect of IGF-I treatment on calf birth weight. In contrast, calves born from recipients that were treated with GnRH on d 11 tended to have higher birth weights. This was true only among all calves born, however, and not for all live calves born. Treatment of cattle with GnRH during the mid-luteal phase can increase blood concentrations of progesterone (Macmillan et al., 1985; Rettmer et al., 1992; Stevenson et al., 1993). Supplemental progesterone during early gestation can significantly increase conceptus length (Garrett et al., 1992), and it may be that the increased birth weights for calves born from GnRH-treated recipients are due to increased circulating concentrations of progesterone during early pregnancy. It is also possible that this result was a statistical aberration since increased birth weights due to GnRH were not observed in the subset of live calves born from GnRH-treated recipients and the number of observations was low (27 calves). Future research is necessary to confirm this observation.

A number of previous studies have reported a skewed sex ratio for calves born following the transfer of in vitro-produced embryos; the percentage of calves that were male has ranged from 55.4 to 82.0% (Massip et al., 1996; Wagtendonk-de Leeuw et al., 1998; Hasler, 2000). Of the 28 calves born in the present study, 18 were males (64.3%). This apparent skewing in the sex ratio did not result because male embryos survived transfer better, but because more embryos were male at the time of transfer. This is evident from the analysis of sex ratio on 105 embryos harvested at d 8 following insemination where 75 (71.4%) were males.

The use of timed embryo transfer has a practical advantage over conventional embryo transfer following detected estrus because it completely eliminates the requirement for estrus detection. However, the effectiveness of timed embryo transfer in establishing pregnancies compared with embryo transfer following detected estrus has not been thoroughly examined. A recent review (Bo et al., 2001) outlines the results of four studies in which pregnancy rates (among all cows treated) following timed embryo transfer with in vivo-derived embryos were similar to or better than those obtained following embryo transfer after detected estrus. Studies involving timed embryo transfer of embryos produced in vitro have been limited to experiments where recipients were exposed to heat stress (Ambrose et al., 1999; Al-Katanani et al., 2002). In the present experiment, overall pregnancy rate at d 53 of gestation in the control recipients was 10.3% (9/87). This is slightly lower than the pregnancy rates previously reported following timed embryo transfer with heat-stressed recipients (14 to 19%; Ambrose et al., 1999; Al-Katanani et al., 2002). One explanation for this difference may be related to differences in culture medium. Fetal calf serum was added to culture medium on d 5 for the two previous studies involving timed embryo transfer with in vitro-produced embryos (Ambrose et al., 1999; Al-Katanani et al., 2002), whereas fetal calf serum was omitted from embryo culture medium in the present experiment. The addition of fetal calf serum likely contains growth factors that are beneficial to embryo development, and these may have increased embryonic viability. In the present study, pregnancy rates at d 53 for IGF-I-treated embryos (22.8% for all recipients and 25.7% for synchronized recipients) were comparable to the results obtained by Ambrose et al. (1999) and Al-Katanani et al. (2002).

In conclusion, this study demonstrates that addition of IGF-I to culture medium increases development of bovine embryos to the blastocyst stage and improves

embryonic survival following transfer to recipients without altering calf birth weight or sex ratio. Future experiments are needed to identify the mechanisms that lead to the increased survival of IGF-I-treated embryos following transfer. The administration of GnRH to recipients on d 11 tended to increase pregnancy and calving rates (to values nearly double the rates observed for control recipients), but differences were not statistically significant. Future research is needed to determine whether the use of GnRH or other hormonal treatments, such as chorionic gonadotropin and progesterone, is an effective means for improving recipient endocrine status and, in turn, increasing embryo survival following transfer to recipients.

## Implications

The addition of insulin-like growth factor-1 to bovine embryo culture medium can increase the proportion of embryos developing to the blastocyst stage. Moreover, transfer of embryos treated with insulin-like growth factor-1 increases the proportion of recipients establishing pregnancy and giving birth to live calves without having a negative effect on calf birth weight and sex ratio. Thus, the incorporation of insulin-like growth factor-1 improves the overall efficacy of in vitro embryo production and transfer. The use of gonadotropin-releasing hormone to improve the fertility of recipients has the potential to increase pregnancy rates, but future research is needed to confirm whether this approach is effective.

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