Effect of treatment with human chorionic gonadotropin 7 days after artificial insemination or at the time of embryo transfer on reproductive outcomes in nulliparous Holstein heifers

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ABSTRACT

Our objective was to assess the effect of treatment with human chorionic gonadotropin (hCG) 7 d after artificial insemination (AI) or at the time of in vitro-fertilized (IVF) embryo transfer on reproductive outcomes, including progesterone (P4), interferon-tau stimulated gene 15 (*ISG15*), pregnancy-specific protein B (PSPB), and pregnancies per AI (P/AI) or pregnancies per embryo transfer (P/ET), in nulliparous Holstein heifers. Heifers in experiment 1 were randomly assigned to receive no treatment (control; n = 129) or 2,000 IU of hCG 7 d after AI to a detected estrus (estrus = experimental d 0; hCG; n = 132). Heifers in experiment 2 were randomly assigned to receive no treatment (control; n = 143) or 2,000 IU of hCG (hCG; n = 148) at transfer of an IVF embryo 7 d after the last GnRH treatment of a 5-d controlled internal drug release-synch protocol (last GnRH = experimental d 0). Blood samples were collected from a subgroup of heifers (experiment 1, n = 82; experiment 2, n = 104) at d 7, 11, 18, 20, 25, 28, and 32, and blood samples from heifers diagnosed pregnant were collected on d 35, 39, 46, 53, 60, and 67. Blood samples were assayed for P4 by RIA and for PSPB by ELISA, and expression of *ISG15* was assessed in mRNA isolated from blood leukocytes on d 18 and 20. Data were analyzed by ANOVA and logistic regression using the MIXED and GLIMMIX procedures. In both experiments, treatment with hCG increased P4 concentrations from d 11 to 32; however, treatment did not affect P/AI or P/ET at d 32 or 67, PSPB concentrations from d 11 to 67 of pregnancy, or relative *ISG15* mRNA concentrations on d 18 or 20. Heifers diagnosed not pregnant at d 32 in experiment 2 with an extended luteal phase (>20 d) and treated with hCG had greater relative *ISG15*

mRNA concentrations on d 20 than control heifers. Treatment with hCG did not affect pregnancy loss in experiment 1, whereas heifers treated with hCG at the time of IVF embryo transfer had fewer pregnancy losses from d 32 to 67 than control heifers. We concluded that treatment with 2,000 IU of hCG 7 d after AI or at the time of embryo transfer increased P4 concentrations without affecting P/AI or P/ET in nulliparous Holstein heifers.

Key words: human chorionic gonadotropin, artificial insemination, embryo transfer, dairy heifer

INTRODUCTION

Embryo transfer (ET) has become increasingly popular in the US dairy industry since it was first commercialized in the 1970s. According to annual reports from the American Embryo Transfer Association (AETA, 2018), the number of fresh and frozen bovine in vitro-fertilized (IVF) embryo transfers increased from 26,742 in 2010 to 117,712 in 2016. Pregnancy loss is a common problem in dairy cattle (Humblot, 2001; Santos et al., 2001) that has substantial economic consequences due to critical delays in time to pregnancy and decreased offspring (Cabrera, 2012), and pregnancy loss after IVF ET is reported to be greater in dairy cows than after AI (Matsuyama et al., 2012).

Progesterone (P4) is a key hormone in the establishment and maintenance of pregnancy in cattle. Embryonic survival is dependent on secretion of interferon-tau (IFN τ) during blastocyst elongation (Mann and Lamming, 2001) and inhibits uterine secretion of PGF $_{2\alpha}$ pulses, thereby preventing luteolysis. Although the effects of exogenous P4 treatment are not consistent among studies, supplementation of P4 from 3 to 13 and 16 d after AI advanced elongation of the conceptus (Carter et al., 2008), which resulted in earlier secretion of IFN τ (Mann et al., 2006) and increased conception rates (Mann and Lamming, 1999). Increasing P4 concentrations in Holstein and Holstein-crossbred heifers by treatment with GnRH 5 d after ovulation did not

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affect pregnancies per ET (**P/ET**), but decreased pregnancy loss for stage 7 embryos (Garcia-Guerra et al., 2015). By contrast, decreased circulating P4 concentrations after ovulation decreased the expression of genes associated with embryo elongation (Forde et al., 2012) and decreased embryo growth (Carvalho et al., 2017).

One method to increase circulating P4 concentrations after AI or ET is by treatment with human chorionic gonadotropin (**hCG**; Price and Webb, 1989; De Rensis et al., 2010). To date, many studies have tested the effect of treatment with hCG after AI on pregnancies per AI (**P/AI**) or pregnancy loss with mixed results. In cows, treatment with hCG on d 5 (Schmitt et al., 1996; Santos et al., 2001; Nascimento et al., 2013), 7 (Sianangama and Rajamahendran, 1992; Dahlen et al., 2010; Wallace et al., 2011; Torres et al., 2013a), and 14 (Sianangama and Rajamahendran, 1992) increased P4 concentrations; however, effects on pregnancy outcomes and pregnancy loss varied. Pregnancy outcomes for heifers treated with hCG have varied even more; P/AI increased after treatment with hCG on d 4 (Breuel et al., 1989) and 7 (Chagas e Silva and Lopes da Costa, 2005; Torres et al., 2013b), whereas no difference was reported in other studies when beef heifers were treated with hCG on d 4 (Breuel et al., 1990), 5 (Funston et al., 2005), or 6 (Funston et al., 2005).

The objectives of our study were to assess the effect of treatment with 2,000 IU of hCG 7 d after AI or at the time of ET on reproductive outcomes, including P4, pregnancy-specific protein B (**PSPB**), interferon-stimulated gene 15 (*ISG15*), and pregnancy outcomes (P/AI or P/ET), in nulliparous Holstein heifers. Our hypotheses were that heifers treated with hCG would have (1) increased P4 concentrations, (2) increased expression of *ISG15* mRNA, and (3) increased serum PSPB concentrations.

MATERIALS AND METHODS

All animal handling and experimental procedures were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the University of Wisconsin–Madison.

Heifers, Housing, and Feeding

Our study was conducted from May to August of 2017 on a commercial dairy herd located near Casco, Wisconsin, that uses both AI and IVF ET on breeding heifers. The farm milks approximately 2,800 Holstein cows and has approximately 700 breeding-age heifers. Height and weight of individual heifers was recorded at birth, weaning, introduction to pens with freestalls, and during transition to the breeding pen (0, 2, 5, and 12

mo, respectively). Heifers weighing 340 kg or more were eligible for insemination beginning at 12 mo of age. All records and measurements were entered and stored in an on-farm dairy management software program (Dairy Comp 305; Valley Agricultural Software, Tulare, CA). Heifers were fed once daily a TMR consisting of grass hay, alfalfa and corn silage as forage with trace distillers, salt, and Rumensin (Elanco, Greenfield, IN). Freestalls were bedded with dried manure solids and all heifers had ad libitum access to feed and water throughout the experiment.

Experiment 1: Artificial Insemination

Nulliparous Holstein heifers ($n = 261$) were AI after a detected estrus, which was evaluated once daily via removal of tail paint. Heifers not detected in estrus 7 d after entering the breeding pen were treated with PGF_{2α} (526 µg of cloprostenol sodium; Estrumate, Merck Animal Health, Kenilworth, NJ) to induce estrus. Heifers were monitored during the next 2 wk for estrus both visually and by tail chalking (Macmillan et al., 1988) and were inseminated once daily in the morning upon detection of rubbed tail chalk. Heifers not detected in estrus, and, therefore not inseminated, received a second PGF_{2α} treatment 14 d after the first. According to the farm protocol, heifers were inseminated with sex-sorted semen for the first 2 inseminations, whereas conventional semen was used for inseminations 3 to 6.

At the beginning of the experiment, any heifers eligible for AI based on the farm protocol were stratified by AI number and were randomized to receive no treatment (control; $n = 129$) or to receive 2,000 IU of hCG (2 mL, Chorulon, Merck Animal Health; $n = 132$) 7 d after AI. Heifers diagnosed not pregnant at the first pregnancy diagnosis after their initial enrollment into the experiment were eligible to be re-enrolled in the experiment and were rerandomized to treatments. The day of estrus was considered to be experimental d 0 (Figure 1A).

Experiment 2: Embryo Transfer

Nulliparous Holstein heifers ($n = 291$) were synchronized for ET using a 5-d controlled internal drug release (**CIDR**) synch protocol (Lima et al., 2013; Figure 1A). Briefly, heifers were treated with GnRH (86 µg i.m.; Fertagyl; Merck Animal Health) and a CIDR (Eazi-Breed CIDR; Zoetis, New York, NY); 5 d later, the CIDR was removed. Heifers were treated with PGF_{2α} (526 µg of cloprostenol sodium; Estrumate, Merck Animal Health) at the time of CIDR removal and 24 h later, and treatment with GnRH occurred 48 h after the second PGF_{2α} treatment. The last GnRH treatment

Figure 1. Schematic diagram of the reproductive protocols used in experiments 1 and 2 (A) and a diagram of sampling for experiments 1 and 2 (B). In experiment 1, the day of estrus was considered experimental d 0; in experiment 2, the last GnRH treatment of the 5-d controlled internal drug release (CIDR)-synch protocol was considered experimental d 0. hCG = human chorionic gonadotropin; P4 = progesterone; PSPB = pregnancy-specific protein B.

of the 5-d CIDR synch protocol was considered experimental d 0 (Figure 1A). Grade 1, 2, or 3 IVF embryos were created and graded according to techniques described by the International Embryo Transfer Society (Stringfellow and Givens, 2010). Seven days after the last GnRH treatment of the 5-d CIDR synch protocol, fresh IVF embryos were transferred into heifers that had a palpable corpus luteum as assessed by an experienced veterinarian who conducted all ET throughout the experiment. According to the farm protocol, heifers received up to 3 successive ET.

At the beginning of the experiment, any heifers eligible for ET based on the farm protocol were stratified by ET number and were randomized to receive no treatment (control; $n = 143$) or to receive 2,000 IU of hCG (2 mL, Chorulon; Merck Animal Health; $n = 148$) at the time of ET (i.e., 7 d after the last GnRH treatment of the 5-d CIDR-synch protocol). Heifers diagnosed not

pregnant at the first pregnancy diagnosis after their initial enrollment into the experiment were eligible to be re-enrolled in the experiment and were rerandomized to treatments. After a nonpregnancy diagnosis, heifers were resynchronized using the same 5-d CIDR synch protocol described for first enrolment.

Pregnancy Diagnosis

For both experiments, pregnancy was diagnosed on $d 32 \pm 3$ d by palpation per rectum, and the pregnancy status of all heifers diagnosed pregnant was reconfirmed by palpation per rectum 35 d later at $d 67 \pm 3$.

Blood Sampling

Blood samples for analysis of P4, PSPB, and *ISG15* mRNA expression in blood leukocytes were collected

from a subgroup of heifers from the initial enrollment into each experiment (experiment 1, $n = 82$ heifers; experiment 2, $n = 104$ heifers). Blood samples for P4 and PSPB analysis were collected via puncture of the median caudal vein or artery into 8-mL evacuated serum collection tubes (Vacuette, Greiner Bio-One North America Inc., Monroe, NC). Blood samples were collected twice weekly beginning on d 7 and continued until d 32; blood samples from heifers diagnosed pregnant on d 32 were collected weekly until d 67 (Figure 2B). Blood samples were placed on ice immediately upon collection and were centrifuged ($1,600 \times g$, 4°C) for 20 min within 2 h of collection. Serum was harvested and stored at -20°C until assayed for P4 and PSPB concentrations. Blood samples (3 mL) for analysis of blood leukocyte *ISG15* mRNA expression were collected on d 18 and 20 from the coccygeal vessels into Tempus Blood RNA tubes containing 6 mL of RNA stabilization solution (Applied Biosystems, Foster City, CA). Tempus tubes were shaken vigorously by hand for 20 s

immediately after blood sample collection, and whole blood was stored in the Tempus tubes at -20°C until the mRNA isolation procedure.

Analysis of P4 and PSPB

Serum P4 concentrations were determined using a solid-phase, no-extraction RIA (MP Biomedicals, Solon, OH). The average sensitivity for the 2 assays was 0.005 ng/mL. The average intra-assay coefficient of variation (CV) was 9.5% and the interassay CV was 6.9% based on a quality control sample (2.50 ng/mL of P4) that was replicated within each P4 assay. Serum PSPB concentrations were determined using a quantitative ELISA (Biopryn, Bio-Tracking LLC, Moscow, ID). The average intra-assay CV for the high- and low-quality control samples was 5 and 6%, respectively, and interassay CV was 5% for both high- and low-quality control sample based on a quality control sample (0.9 ng/mL of PSPB) that was replicated within each PSPB assay.

Analysis of ISG15

Total RNA was extracted from blood leukocytes using the Tempus Spin RNA Isolation Kit (Applied Biosystems) as per the manufacturer's instructions (<http://tools.thermofisher.com/content/sfs/manuals/4382075b.pdf>), including the optional DNase step. Extracted RNA (1 μg) was reverse transcribed to cDNA (Bio-Rad iScript Reverse Transcription Supermix #1708840; Bio-Rad, Hercules, CA). Quantitative real-time PCR was conducted with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Reaction mixtures and cycling conditions were performed as described elsewhere (Carvalho et al., 2017). Amplification efficiencies of primers were accepted within a range of 95 to 105% efficiency, and primer specificity was assessed by the presence of a single temperature dissociation peak. Cyclophilin A (NM_178320; forward 5'-CACCGTGTTCTTCGACATCG-3', reverse 5'-ACAGCTCAAAAGAGACGCGG-3') was used as a housekeeping gene, and all samples were analyzed for *ISG15* (NM_174366; forward 5'-GGTATGAGCTGAAGCAGTT-3', reverse 5'-ACCTCCCTGCTGTCAAGGT-3'). Analysis was conducted using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001).

Statistical Analyses

All statistical analyses were performed using SAS computational software version 9.4 for Microsoft Windows (SAS Institute Inc., Cary, NC). Analyses of binary response data (P/AI, P/ET, and pregnancy loss) were

Figure 2. Mean ($\pm$ SEM) serum progesterone concentrations for heifers that maintained pregnancy through d 67 in experiment 1 (upper panel) and experiment 2 (lower panel). hCG = human chorionic gonadotropin.

Table 1. Effect of treatment on mean ($\pm$ SEM) progesterone (ng/mL) concentrations in heifers that maintained pregnancy in experiment 1¹

Day	Control (n = 16)	hCG (n = 16)	Difference	Increase (%)	P-value
7	4.3 $\pm$ 0.3	4.2 $\pm$ 0.6	-0.1	—	0.92
11	6.2 $\pm$ 0.5	9.1 $\pm$ 0.9	+2.9	47	<0.01
18 to 67	9.2 $\pm$ 0.3	14.7 $\pm$ 0.4	+5.4	59	<0.01

¹Seven days after AI, heifers were randomly assigned to receive no treatment (control) or 2,000 IU of human chorionic gonadotropin (hCG).

performed by logistic regression using the GLIMMIX procedure of SAS. Selection of the model that best fit the data for each variable of interest was performed by finding the model with the lowest value for the Akaike information criterion using a backward elimination procedure that removed all variables with $P > 0.10$ from the model. The fixed effect of treatment was forced to remain in each model. The initial model for experiment 1 included the fixed effects of semen type (sex-sorted vs. conventional), treatment, AI number, and the 2-way interactions; the final model included the fixed effects of semen type and treatment. The initial model for experiment 2 included the fixed effects of embryo stage (stage 4, 5, 6, 7, and 8), grade (grade 1, 2, or 3), ET number, treatment, and the 2-way interactions; the final model included the fixed effects of embryo grade and treatment. Heifer was the experimental unit and each AI or ET was considered to be an independent event.

Concentrations of P4 and PSPB were analyzed by ANOVA using the MIXED procedure of SAS. The model contained the fixed effects of treatment, time, and the 2-way interaction. Due to repeated measures within each heifer, a repeated statement was used to account for autocorrelations by modeling the spatial power between sequential measurements. Concentrations of *ISG15* mRNA on d 18 and 20 were analyzed by ANOVA using the MIXED procedure of SAS. The model contained the fixed effects of treatment, pregnancy status, and time after the stepwise elimination of nonsignificant interactions. Data from the *ISG15* mRNA analysis were further analyzed by generating receiver operating characteristic (ROC) curves using

the LOGISTIC procedure in SAS to determine a cutoff threshold for a positive *ISG15* mRNA expression indicative of pregnancy. Only nonpregnant heifers with a normal luteal phase of 18 d (i.e., true nonpregnant) and heifers diagnosed pregnant (i.e., true pregnant) were used to generate ROC curves to establish *ISG15* mRNA pregnancy cutoffs. The model contained the fixed effect of pregnancy and *ISG15* mRNA expression as a variable.

A significant difference between levels of a classification variable was declared when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were declared a statistical tendency. Data are presented as least squares means $\pm$ standard error of the means obtained from the MIXED procedure of SAS.

RESULTS AND DISCUSSION

Effect of Treatment on Serum P4 Concentrations

In the present study, heifers treated with hCG that maintained pregnancy through d 67 had greater plasma P4 concentrations than pregnant control heifers ($P \leq 0.01$) from d 11 to 67 of pregnancy in both experiment 1 (Figure 2, upper panel) and 2 (Figure 2, lower panel). Serum P4 concentrations did not differ between treatments in either experiment on d 7; however, P4 concentrations were about 1 ng/mL greater on d 7 in experiment 1 versus 2 (4.26 vs. 3.31 ng/mL; Tables 1 and 2). Treatment with hCG increased ($P < 0.01$) P4 concentrations on d 11 (47 vs. 58% increase for experiment 1 vs. 2, respectively) and from d 18 to 67 (59 vs.

Table 2. Effect of treatment on mean ($\pm$ SEM) progesterone (ng/mL) concentrations in heifers that maintained pregnancy in experiment 2¹

Day	Control (n = 15)	hCG (n = 25)	Difference	Increase (%)	P-value
7	3.5 $\pm$ 0.3	3.1 $\pm$ 0.3	-0.4	—	0.42
11	5.5 $\pm$ 0.4	8.7 $\pm$ 0.7	+3.2	58	<0.01
18 to 67	8.1 $\pm$ 0.3	12.2 $\pm$ 0.3	+4.1	50	<0.01

¹Heifers were randomly assigned to receive no treatment (control) or 2,000 IU of human chorionic gonadotropin (hCG) at transfer of an IVF embryo 7 d after the last GnRH treatment of a 5-d controlled internal drug release-synch protocol.

50% increase for experiment 1 vs. 2, respectively) in both experiments (Tables 1 and 2).

Progesterone produced by the corpus luteum plays a critical role in the establishment and maintenance of pregnancy in cattle (Mann and Lamming, 1999). Conception failure and poor embryonic development can result from prolonged low circulating P4 between 5 and 10 d after insemination (Mann et al., 1999; Thatcher et al., 2001). The objective of the present experiment was to increase P4 after AI or ET by treatment with hCG. We chose to treat heifers with hCG on d 7 rather than d 5 of the estrous cycle to induce ovulation of a larger follicle, thereby forming a larger corpus luteum that produces more P4 (Vasconcelos et al., 2001). Although we did not assess ovulatory response to hCG or formation of accessory corpus luteum in these experiments, we expected a high ovulatory response to hCG treatment administered on d 7 of the estrous cycle because a first-wave dominant follicle would be present. The homology in β -subunits between hCG and bovine LH is ~80% (Pierce and Parsons, 1981); thus, treatment with hCG has an LH-like effect in cattle, resulting in an increase in serum P4 concentration either by stimulating an increase in P4 production from the existing corpus luteum (Stevenson et al., 2007), by inducing ovulation of a dominant follicle to form an accessory corpus luteum (Schmitt et al., 1996; Santos et al., 2001; Thatcher et al., 2001; Nascimento et al., 2013), or via both mechanisms. In other experiments, treatment with hCG during the early (d 4 to 7) luteal phase when a dominant follicle of the first follicular wave is present caused formation of an accessory corpus luteum in 83 (Price and Webb, 1989), 97 (Torres et al., 2013b), and 100% (Diaz et al., 1998) of nulliparous Holstein heifers.

Effect of Pregnancy Status on Circulating P4

Heifers diagnosed pregnant on d 32 in experiment 1 had similar P4 concentrations to heifers diagnosed not pregnant on d 7 and 11, but greater ($P < 0.01$) serum P4 than heifers diagnosed not pregnant beginning on d 18 (Figure 3, upper panel); this difference continued through d 32 (13.1 ± 1.0 vs. 4.4 ± 0.9 ng/mL, pregnant vs. open). The decrease in serum P4 on d 18 in heifers diagnosed not pregnant was due to luteolysis (Mann et al., 1999). Differences in P4 due to pregnancy status before luteolysis in the present study are in agreement with others. When heifers were inseminated to a detected estrus, P4 concentrations did not differ between pregnant and nonpregnant cows and heifers 7 d after AI (Santos et al., 2001; Chagas e Silva and Lopes da Costa, 2005) or ET (Niemann et al., 1985; Stubbings and Walton, 1986; Chagas e Silva et al., 2002; Chagas e Silva and Lopes da Costa, 2005; Torres et al., 2013b).

In contrast to experiment 1, heifers diagnosed pregnant at d 32 in experiment 2 had greater ($P < 0.01$) serum P4 at ET (i.e., 7 d after the second GnRH treatment) than heifers diagnosed not pregnant (Figure 3, lower panel). The difference in pregnancy outcomes based on P4 concentrations on the day of ET adds to this often reported yet poorly understood observation (Dahlen et al., 2010; Pereira et al., 2013). Dahlen et al. (2010) reported that pregnant beef cows had greater P4 concentrations than nonpregnant cows 7 d after fixed-time AI after a Co-Synch + CIDR protocol (Larson et al., 2006). In addition, Pereira et al. (2013) reported that lactating dairy cows with P4 < 1.55 ng/mL on the day of ET were more likely to undergo pregnancy loss from 28 to 60 d. In the present study, we noted a tendency ($P = 0.06$) for P4 concentrations to be greater in heifers that maintained pregnancy until d 67 than in heifers that underwent pregnancy loss from d 32 to 67 (data not shown). By contrast, Pereira et al. (2016) reported P4 concentrations on d 7 were associated with P/AI but not P/ET in lactating dairy cows. In addition, Stubbings and Walton (1986) reported that P4 concentrations at ET were not related to pregnancy

Figure 3. Effect of pregnancy status at d 32 on mean ($\pm$ SEM) serum progesterone concentrations on d 7, 11, and 18 in experiment 1 (upper panel) and experiment 2 (lower panel).

status in Holstein-Friesian heifers; however, pregnancy status was not assessed until 60 d in that study.

Overall, it is possible that the observed difference in pregnancy outcomes was due to ovulation of a smaller follicle in heifers submitted to a 5-d CIDR synch protocol. Ovulation of a smaller follicle results in a smaller corpus luteum that secretes less P4 after AI in lactating dairy cows (Vasconcelos et al., 2001). This idea agrees with the lower initial P4 concentrations of heifers that were synchronized or resynchronized using a 5-d CIDR synch protocol for ET in experiment 2 than of heifers inseminated to a detected estrus in experiment 1 in the present study (Tables 1 and 2). Further research is needed to understand how and why pregnancy outcomes are related to P4 concentrations early after the second GnRH treatment for heifers receiving IVF embryos.

Effect of Treatment on Pregnancy Outcomes and Pregnancy Loss

Of the 261 pregnancy outcomes after AI analyzed in experiment 1, data from 224 AI (86%) were from heifers initially enrolled into the experiment, whereas 37 AI (14%) were from heifers diagnosed not pregnant after first enrolment that were subsequently rerandomized to treatments and enrolled into experiment 1 a second time. Mean ($\pm$ SEM) AI number for heifers initially enrolled into experiment 1 was 1.98 ± 0.09 (range = 1 to 6), and mean ($\pm$ SEM) AI number for heifers re-enrolled into experiment 1 was 2.95 ± 0.19 (range = 2 to 6). Of the 291 pregnancy outcomes after ET analyzed in experiment 2, data from 249 ET (86%) were from heifers initially enrolled into the experiment, whereas 42 ET (14%) were from heifers diagnosed not pregnant after first enrolment that were subsequently rerandomized to treatments and enrolled into experiment 2 a second time. Mean ($\pm$ SEM) ET number for heifers initially enrolled into experiment 2 was 1.37 ± 0.04 (range = 1 to 3), and mean ($\pm$ SEM) ET number for heifers re-enrolled into experiment 2 was 2.33 ± 0.07 (range

= 2 to 3). Neither AI number (experiment 1) nor ET number (experiment 2) differed between treatments or affected pregnancy outcomes or pregnancy loss.

Overall, P/AI and P/ET did not differ between treatments for heifers in both experiment 1 (Table 3) and experiment 2 (Table 4) at either d 32 or 67. We did note, however, an effect of semen type on P/AI in experiment 1 (Table 3). Experiment 1 was not designed to directly test the effect of semen type on fertility because semen type was confounded with insemination number. Nonetheless, P/AI in experiment 1 was affected by semen type, in which P/AI to sex-sorted semen was 55% that of heifers inseminated using conventional semen (Table 3). The observed difference in fertility of heifers inseminated using sex-sorted versus conventional semen in the present study was greater than the 70 to 85% of the fertility of conventional semen reported by others (Bodmer et al., 2005; DeJarnette et al., 2009; Chebel et al., 2010; Seidel, 2014). Decreased fertility of heifers inseminated with sex-sorted semen can be explained in part by the difference in the number of sperm per straw (2.1×10^6 vs. $15\text{--}20 \times 10^6$ sperm, sexed vs. conventional) and damage to sperm that occurs during the sorting process. Furthermore, timing of insemination relative to ovulation in experiment 1 was not optimized because detection of estrus was performed once daily based on rubbed tail chalk. The optimal timing of AI relative to ovulation may differ for sex-sorted semen due to the decreased lifespan of sperm in the female reproductive tract resulting from the sorting process (Sá Filho et al., 2010; Gosálvez et al., 2011; Sales et al., 2011). More P/AI occurred when insemination with sex-sorted semen occurred 60 versus 54 h after CIDR removal (Sales et al., 2011) as well as 16.1 to 24 (Sá Filho et al., 2010) and 13 to 41 h (Bombardelli et al., 2016) after the onset of estrus. By contrast, inseminating dairy heifers with conventional semen immediately after a detected estrus resulted in similar conception rates compared with strict use of the am–pm rule (Wahome et al., 1985). The effect of treatment on P/AI and P/ET (Tables 3 and 4) should be interpreted with caution because of

Table 3. Effect of treatment and semen type [% (no./no.)] on pregnancies per artificial insemination (P/AI) and pregnancy loss for Holstein heifers in experiment 1

Day	Treatment ¹		Semen type ²		P-value	
	Control	hCG	CS	SX	Treatment	Semen
32	41 (53/129)	43 (57/132)	62 (46/74)	34 (64/187)	0.96	<0.01
67	41 (53/129)	41 (54/132)	61 (45/74)	33 (62/187)	0.66	<0.01
Loss ³	0 (0/53)	5 (3/57)	2 (1/46)	3 (2/64)	0.97	0.62

¹Seven days after AI, heifers were randomly assigned to receive no treatment (control) or 2,000 IU of human chorionic gonadotropin (hCG).

²CS = heifers inseminated with conventional semen; SX = heifers inseminated with sex-sorted semen.

³Heifers diagnosed pregnant 32 d after AI that were diagnosed not pregnant 67 d after AI.

the limited number of heifers per treatment enrolled into each experiments.

Overall, hCG treatment did not affect pregnancy outcomes (P/AI or P/ET) at d 32 or 67 for heifers in both experiments. Effects of hCG treatment 7 d after AI on fertility have varied among studies. No difference in P/AI was observed in beef heifers when treated with hCG 4 d after AI (Breuel et al., 1990), and at 2 out of 3 locations 5 and 6 d after AI (Funston et al., 2005) or 7 d after AI (Chagas e Silva and Lopes da Costa, 2005) in dairy heifers. An effect of parity on fertility has been reported in lactating dairy cows in which treatment with hCG 5 d after TAI increased fertility in primiparous cows but did not increase fertility in multiparous cows (Nascimento et al., 2013). Considering that treatment with hCG has been reported to affect fertility based on parity, it is possible that the high fertility observed in nulliparous heifers raised under optimal conditions in the present study may not benefit from treatment with hCG.

In experiment 1, treatment with hCG did not affect pregnancy loss from d 32 to 67 when heifers were inseminated to a detected estrus (Table 3). An interesting observation in experiment 2 was the effect of treatment on pregnancy loss from d 32 to 67 ($P = 0.04$), in which heifers treated with hCG had fewer pregnancy losses than control heifers (Table 4). This observation should be interpreted with caution because this experiment was not statistically powered to assess pregnancy loss. None of the heifers diagnosed pregnant after AI in experiment 1 lost pregnancies from d 67 to calving, whereas 5 heifers in experiment 2 diagnosed pregnant after IVF ET lost pregnancies from d 67 to calving (3 control heifers and 2 hCG heifers).

Although studies evaluating the effect of hCG on pregnancy loss for heifers receiving ET are limited, treatment with GnRH 5.5 d after ovulation decreased pregnancy loss in stage 7 embryos for Holstein and Holstein-cross Heifers (Garcia-Guerra et al., 2015). Chagas e Silva and Lopes da Costa (2005) reported no effect of hCG treatment on d 7 on P/ET in dairy heifers;

however, pregnancy loss was not reported in that study. In addition, treatment with hCG 5 d after ovulation did not affect P/ET for lactating Holstein cows receiving ET 7 d after induction of ovulation (Galvão et al., 2006). In mature beef cows, treatment with hCG at ET 7 d after ovulation increased P/ET, but no difference was noted in pregnancy loss (Wallace et al., 2011). Similarly, treatment of Black Japanese cows with hCG 6 d after induction of ovulation increased P/ET versus treatment with hCG 1 d after ovulation and in non-treated controls (Nishigai et al., 2002). Further experiments are needed to support or refute our observation that treatment with 2,000 IU of hCG at the time of ET decreases pregnancy loss.

Developmental stage of transferred embryos did not affect P/ET in the present study (data not shown); however, P/AI and pregnancy loss was affected by embryo grade, in which grade 1 embryos resulted in more P/ET than transfer of grade 2 or grade 3 embryos (Table 4). In addition, heifers receiving grade 1 embryos tended ($P = 0.09$) to have fewer pregnancy losses than heifers receiving grade 3 embryos (Table 4). Embryos are graded by evaluating stage of development and detectable defects. The decrease in P/ET with increasing embryo grade has been consistently reported by others (Hasler, 2001; Chebel et al., 2008).

Effect of Treatment on Serum PSPB Concentrations

Pregnancy-specific protein B concentrations have been used as an indicator of pregnancy (Sasser et al., 1986) and pregnancy loss (Giordano et al., 2012). Experimentally decreasing serum P4 after AI in lactating dairy cows decreased PSPB concentrations by decreasing embryonic growth (Carvalho et al., 2017), whereas increasing serum P4 can advance embryonic growth by increasing endometrial gene expression responsible for histotroph composition (Forde et al., 2009). In the present experiments, we evaluated the effect of increasing P4 after AI or ET on trophoblast growth based on serum PSPB concentrations. Figure 4 shows the effect

Table 4. Effect of treatment and embryo grade [% (no./no.)] on pregnancies per embryo transfer (P/ET) and pregnancy loss for Holstein heifers in experiment 2

Day	Treatment ¹		Embryo grade			P-value	
	Control	hCG	1	2	3	Treatment	Grade
32	48 (68/143)	43 (63/148)	54 ^a (95/176)	37 ^b (33/89)	12 ^c (3/26)	0.47	<0.001
67	37 (53/143)	39 (57/148)	47 ^a (82/176)	30 ^b (27/89)	4 ^c (1/26)	0.67	0.001
Loss ²	22 (15/68)	10 (6/63)	14 (13/95)	18 (6/33)	67 (2/3)	0.04	0.09

^{a-c}Within a row, percentages with different superscripts differ ($P < 0.05$).

¹Seven days after the second GnRH treatment, heifers were randomly assigned to receive 2,000 IU of human chorionic gonadotropin (hCG) or remain untreated (control).

²Heifers diagnosed pregnant 32 d after ovulation that were diagnosed not pregnant 67 d after ovulation.

Figure 4. Mean ($\pm$ SEM) serum pregnancy-specific protein B (PSPB) concentrations in heifers that maintained pregnancy through d 67 in experiment 1 (upper panel) and experiment 2 (lower panel). hCG = human chorionic gonadotropin.

of treatment on serum PSPB profiles for heifers that maintained pregnancy until d 67 in experiment 1 (Figure 4: upper panel) and experiment 2 (Figure 4: lower panel). In both experiments, serum PSPB concentrations in heifers were low on d 18 and 20 then increased until they peaked on d 35 in experiment 1 or d 39 in experiment 2. Overall, treatment did not affect serum PSPB concentrations for pregnant heifers in either experiment, nor was a treatment by time interaction detected.

The lack of an effect of treatment on PSPB concentrations in the present studies suggests that P4 concentrations were not limiting trophoblast growth in heifers that maintained pregnancy after AI or ET until d 67. Exogenous supplementation of P4 from 7 to 19 d after ET increased PSPB concentrations for heifers receiving demi-embryos beginning 25 d after estrus, whereas heifers receiving whole embryos had a less pronounced PSPB increase from 49 to 63 d (Lopes-da-Costa et al., 2011). Thus, smaller and less viable embryos may benefit from increased P4 early after transfer to compensate for their smaller initial size. Temporarily decreasing P4 after timed AI in lactating dairy cows decreased PSPB concentrations through the first 67 d of pregnancy and

embryonic growth (Carvalho et al., 2017). Data from the present study suggest that the opposite is not necessarily true in nulliparous Holstein heifers. In heifers, PSPB has a higher sensitivity for a positive pregnancy status on d 24 and 26 of pregnancy than in cows (Humboldt et al., 1988). Increasing P4 by treatment with hCG in the present study had no effect on PSPB concentrations; this can partially explain why hCG does not appear to benefit highly fertile animals (Santos et al., 2001), or it may indicate that P4 is not the rate-limiting factor for embryonic development in heifers once P4 reaches adequate concentrations in circulation.

Effect of Treatment on *ISG15* mRNA Expression

The bovine blastocyst initiates the production and release of a paracrine signal, $\text{IFN}\tau$, to prevent secretion of uterine $\text{PGF}_{2\alpha}$ pulses, thereby preventing luteolysis and initiating maternal recognition of pregnancy (Spencer and Bazer, 2004). Produced by the conceptus, $\text{IFN}\tau$ is detectable in d 7 bovine blastocysts (Farin et al., 1990; Kubisch et al., 1998) and is dramatically up-regulated concomitant to blastocyst elongation (Mann and Lamming, 1999; Mann and Lamming, 2001; Rizos et al., 2012) due to its role in the regulation of genes that play pivotal roles in implantation and conceptus growth (Spencer et al., 2007). The presence of $\text{IFN}\tau$ can be detected indirectly through upregulation of several IFN -induced genes, including *ISG15* (Han et al., 2006). Finally, an experiment directly comparing gene expression by in vivo- and in vitro-produced d 7 and 16 embryos reported that the in vitro production of bovine embryos negatively affected the amount of gene expression on d 7 and the rate of development on d 16 (Bertolini et al., 2002). Thus, reproductive outcomes may differ based on embryo type.

Figure 5 illustrates the effect of treatment and pregnancy outcome on relative expression of *ISG15* mRNA for heifers in experiment 1 (Figure 5, upper panel) and experiment 2 (Figure 5, lower panel). As expected, we observed an effect of pregnancy status ($P < 0.01$) on *ISG15* mRNA expression for heifers in both experiments, in which pregnant heifers had greater *ISG15* mRNA expression than nonpregnant heifers. In experiment 1, we found no effect of treatment or time after treatment with hCG on d 7. Interestingly, high relative *ISG15* mRNA expression was detected in 1 heifer that underwent pregnancy loss between d 32 and 67 on both d 18 and 20 (13.9 and 9.8, respectively), and we speculated that loss of this pregnancy may have been due to twins (data not shown). In experiment 2, we observed no effect of treatment with hCG at the time of ET on *ISG15* mRNA expression; however, we did detect an effect of day in which *ISG15* mRNA expression increased

($P < 0.01$) in heifers from d 18 to 20. In addition, there was no treatment by pregnancy status interaction on relative *ISG15* mRNA expression.

Nonpregnant Heifers

Because heifers in experiment 2 treated with hCG at the time of ET had fewer pregnancy losses from d 32 to 67, we sought to further analyze potential effects of hCG treatment earlier after ET in heifers diagnosed not pregnant on d 32. Further, treatment of heifers with hCG at the time of ET may have altered follicular dynamics later during the estrous cycle. The second follicular wave of an estrous cycle begins earlier in nulliparous heifers than in lactating dairy cows (Ginther et al., 1989; Sartori et al., 2004). Treatment with hCG on d 7 induces ovulation of the first wave dominant follicle in a high proportion of heifers (Schmitt et al., 1996), thereby delaying luteolysis by altering follicular dynamics near the time of luteolysis. Heifers with 3 follicular waves tend to have longer interovulatory intervals than heifers with 2 follicular waves (Ginther et al.,

1989) due to the role of estradiol in luteolysis in which the timing of estradiol secretion regulates the timing of the increased expression of uterine oxytocin receptors and subsequent uterine $\text{PGF}_{2\alpha}$ secretion (Araujo et al., 2009).

The day of luteolysis was determined for individual heifers in experiments 1 and 2 diagnosed not pregnant and was defined as the day P4 concentration decreased to <1.0 ng/mL, as described elsewhere (Ricci et al., 2017). In experiment 1, only 10% of hCG-treated versus 50% of control heifers underwent luteolysis on d 18 (i.e., normal luteal phase; Figure 6, upper panel), whereas 40% of hCG-treated versus 15% of control heifers underwent luteolysis on d 25 (i.e., increased luteal phase). Thus, treatment of heifers with hCG in experiment 1 increased the interovulatory interval, likely by inducing ovulation of the first wave dominant follicle, thereby increasing the occurrence of 3-wave estrous cycles.

By contrast, more variability in the timing of luteolysis for heifers after treatment with hCG was observed in experiment 2 (Figure 6, lower panel). Similar to experiment 1, we noted a tendency ($P < 0.09$) for more control heifers to undergo luteolysis on d 18 (48 vs. 20%, respectively). In contrast to experiment 1, a

Figure 5. Effect of treatment and pregnancy status on d 32 on mean ($\pm$ SEM) relative *ISG15* mRNA on d 18 and 20 in experiment 1 (upper panel) and experiment 2 (lower panel). hCG = human chorionic gonadotropin.

Figure 6. Day of luteolysis (progesterone <1.0 ng/mL) for heifers diagnosed not pregnant on d 32 in experiment 1 (upper panel) and experiment 2 (lower panel). hCG = human chorionic gonadotropin; NR = no regression. *Indicates a treatment difference within a day ($P < 0.01$); **indicates a tendency for a treatment difference within a day ($0.05 \leq P < 0.09$).

subgroup of control heifers [14.8% (4/27)] and heifers treated with hCG [21.1% (4/19)] failed to undergo luteolysis (i.e., P4 never decreased to <1 ng/mL during the sampling period) even though these heifers were diagnosed not pregnant on d 32. This observation supports the idea that although IVF embryos are capable of signaling maternal recognition of pregnancy, as evidenced by extending luteal phases, some of the IVF embryos in the present study did not survive until the pregnancy diagnosis on d 32. Indeed, treatment with hCG at the time of ET in experiment 2 did not affect P/ET on d 32. Our results agree with other studies in which lactating dairy cows receiving IVF embryos had high P4 21 d after ET but lower P/ET at a pregnancy diagnosis conducted later (Ambrose et al., 1999; Block and Hansen, 2007).

Nonpregnant heifers in both experiments were further grouped by luteal phase duration (≤ 20 vs. ≥ 25 d), and heifers diagnosed pregnant on d 32 were grouped by whether they lost or maintained pregnancy from d 32 to 67 to analyze the effect of hCG treatment on mean relative *ISG15* mRNA expression on d 20 in heifers from experiment 1 (Figure 7, upper panel) and experiment 2 (Figure 7, lower panel). In experiment 1, relative *ISG15* mRNA expression did not differ between heifers with normal (≤ 20 d) or extended (≥ 25 d) luteal phases, suggesting that extended luteal phases in experiment 1 were not due to the presence of IFN γ secretion from elongated embryos. To further analyze these data, ROC curves were generated to establish the *ISG15* cutoff level predictive of pregnancy in both experiments. The effect of treatment on the proportion of nonpregnant heifers and pregnant heifers with *ISG15* mRNA concentrations above the pregnancy cutoff threshold are shown in Tables 5 and 6.

Heifers in experiment 2 with normal luteal phases did not have increased *ISG15* mRNA expression. Approximately 20 to 50% of pregnancy loss in high-producing dairy cows occurs during the first week of

Figure 7. Mean ($\pm$ SEM) relative *ISG15* mRNA expression on d 20 in experiment 1 (upper panel) and experiment 2 (lower panel). Nonpregnant heifers were grouped by luteal phase duration (d ≤ 20 vs. d ≥ 25), whereas heifers diagnosed pregnant on d 32 were grouped based on whether they underwent pregnancy loss (loss by d 67) or maintained pregnancy (pregnant). hCG = human chorionic gonadotropin.

pregnancy (Wiltbank et al., 2016); thus, we would not expect pregnancy loss before maternal recognition of pregnancy to affect luteal phase duration. By contrast, heifers in experiment 2 with extended luteal phases and treated with hCG had greater ($P = 0.01$) *ISG15* mRNA expression than control heifers, suggesting that ex-

Table 5. Percentage (no./no) of heifers with positive *ISG15* mRNA expression indicative of pregnancy for nonpregnant heifers in experiment 1 with normal (≤ 20 d) or extended (25 to 32 d) luteal phase, and heifers diagnosed pregnant on d 32 that underwent pregnancy loss (loss) or maintained pregnancy until d 67 (pregnant)¹

Item	d 18		d 20	
	Control	hCG	Control	hCG
Nonpregnant				
≤ 20 d	29 (5/17)	17 (1/6)	27 (3/11)	20 (1/5)
25 to 32 d	100 (3/3)	31 (2/13)	67 (2/3)	31 (4/13)
Pregnant				
Loss	—	50 (1/2)	—	50 (1/2)
Pregnant	75 (12/16)	82 (14/17)	94 (15/16)	88 (14/16)

¹Seven days after AI, heifers were randomly assigned to receive no treatment (control) or 2,000 IU of human chorionic gonadotropin (hCG).

Table 6. Percentage (no./no.) of heifers with positive *ISG15* mRNA expression indicative of pregnancy for nonpregnant heifers in experiment 2 with normal (≤ 20 d) or extended (≥ 25 d) luteal phases, and heifers diagnosed pregnant on d 32 that underwent pregnancy loss (loss) or maintained pregnancy until d 67 (pregnant)¹

Item	d 18		d 20	
	Control	hCG	Control	hCG
Nonpregnant				
≤ 20 d	38 (6/16)	12 (1/8)	33 (4/12)	0 (0/7)
25 d–NR ²	64 (7/11)	30 (3/10)	55 (6/11)	64 (7/11)
Pregnant				
Loss	75 (6/8)	100 (3/3)	75 (6/8)	100 (3/3)
Pregnant	72 (10/14)	83 (20/24)	100 (15/15)	96 (22/23)

¹Seven days after the second GnRH treatment, heifers were randomly assigned to receive 2,000 IU of human chorionic gonadotropin (hCG) or remain untreated (control).

²NR = no regression (progesterone >1.0 ng/mL).

tended luteal phases for heifers receiving IVF embryos and treated with hCG may have occurred due to the presence of elongated embryos producing enough IFN τ to signal maternal recognition of pregnancy. Expression of *ISG15* mRNA can be increased by supplementation of intrauterine IFN τ (Matsuyama et al., 2012) and by increasing P4 in crossbred beef heifers (Forde et al., 2009), whereas lactating cows in which P4 was temporarily decreased after AI had decreased *ISG15* mRNA expression (Carvalho et al., 2017). Our results are similar to a study by Matsuyama et al. (2012) in which cows with an extended luteal phase after ET had increased relative *ISG15* mRNA expression on d 18. Taken together, these observations suggest that pregnancy is not consistently established even when IFN τ is secreted by an elongated embryo. One possibility is that the low expression of *ISG15* mRNA detected is indicative of insufficient growth and low viability of embryos, which then die after initiating maternal recognition of pregnancy. In addition, the quantity of IFN τ may have been insufficient to induce endometrial genes needed to support early embryonic growth (Spencer et al., 2007). Nevertheless, both early and late embryonic mortality are the greatest hurdles to overcome to further increase the adoption of IVF ET in the dairy industry. Results from experiment 2 support a role for treatment with hCG at the time of transfer of an IVF embryo to increase P4 and possibly to decrease pregnancy loss in nulliparous Holstein heifers. Larger studies are needed to corroborate the observation from the present study that treatment with 2,000 IU of hCG at the time of IVF ET decreases pregnancy loss in nulliparous Holstein heifers.

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