Temporarily decreasing progesterone after timed artificial insemination decreased expression of interferon-tau stimulated gene 15 (*ISG15*) in blood leukocytes, serum pregnancy-specific protein B concentrations, and embryo size in lactating Holstein cows

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ABSTRACT

Our objective was to evaluate the effects of temporarily decreasing progesterone (P4) after timed artificial insemination (TAI) on embryonic growth in dairy cows. Lactating Holstein cows ($n = 80$) were submitted to a Double-Ovsynch protocol for first TAI and were assigned randomly to receive 12.5 mg of PGF_{2α} 5 d after the last GnRH treatment (LowP4) or remain untreated (control). Blood samples were collected thrice weekly from 5 to 29 d after TAI for all cows and from 32 to 67 d for pregnant cows, and were analyzed for P4 and pregnancy-specific protein B concentrations. Expression of interferon-tau stimulated gene 15 (*ISG15*) was assessed in blood leukocyte mRNA 18 and 20 d after TAI. Pregnancy diagnosis was performed weekly using ultrasound from 32 to 67 d after TAI, and embryonic crown-rump length was measured 32, 39, and 46 d after TAI. Data were analyzed by ANOVA and logistic regression using the MIXED and GLIMMIX procedures of SAS. The LowP4 cows had less P4 than control cows from 6 to 11 d after TAI; however, pregnancy outcomes 32 d after TAI and pregnancy loss from 32 to 67 d after TAI did not differ between treatments. Control cows diagnosed pregnant 32 d after TAI had greater expression of *ISG15* 20 d after TAI than LowP4 cows diagnosed pregnant 32 d after TAI, and pregnant control cows had greater pregnancy-specific protein B concentrations from 25 to 67 d after TAI than pregnant LowP4 cows. Embryo size did not differ between treatments 32 and 39 d after TAI, but control cows had larger embryos 46 d after TAI. In conclusion, temporarily decreasing P4 after TAI decreased embryonic growth during early pregnancy in lactating Holstein cows but did not affect pregnancies per artificial insemination or pregnancy loss.

Key words: progesterone, interferon-tau stimulated gene 15, pregnancy-specific protein B, embryo size

INTRODUCTION

A key hormone involved in control of the uterine environment is progesterone (P4; Ashworth and Bazer, 1989; Forde et al., 2009, 2012). Progesterone after AI is unequivocally required to support embryo development as well as for establishment and maintenance of pregnancy (reviewed by Spencer et al., 2016). Lactating Holstein cows have lower circulating P4 concentrations than nonlactating Holstein heifers (Sartori et al., 2004), and decreased P4 concentrations in lactating cows have been attributed to increased hepatic steroid metabolism associated with high feed intake (Sangsritavong et al., 2002; Vasconcelos et al., 2003; Wiltbank et al., 2006). In this regard, supplementation with exogenous P4 increases circulating P4 concentrations and induces changes in the endometrial transcriptome (Forde et al., 2009; Carter et al., 2010), leading to increased embryo elongation on d 13 to 16 of pregnancy (Carter et al., 2008; Clemente et al., 2009). By contrast, decreasing P4 concentrations decreased the abundance of mRNA for genes related to embryo elongation (Forde et al., 2011, 2012).

Based on these data, supplementation of cows with exogenous P4 after AI may increase fertility as measured by pregnancies per AI (P/AI). In vivo studies evaluating the effects of P4 supplementation after AI on P/AI or pregnancies per embryo transfer, however, do not consistently support this idea. Results from various studies have reported an increase (Monteiro et al., 2014), no change (Stevenson et al., 2007; Monteiro et al., 2015), or a decrease (Parr et al., 2014) in P/AI for cows supplemented with exogenous P4 after ovulation compared with untreated controls. Although several studies evaluated the effect of decreasing P4 concentrations after ovulation on the uterine environment and embryo development (Forde et al., 2011, 2012), to our

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knowledge, only one study has evaluated the effect of decreasing P4 after ovulation on establishment of pregnancy (Kenyon et al., 2013). In that study, however, no pregnancies occurred after transfer of embryos into the uterus of cows manipulated to have low P4 concentrations.

Treatment with 12.5 mg of PGF_{2α} 5 d after induction of ovulation induced a temporary decrease in P4 concentrations in lactating dairy cows without inducing complete luteal regression, which was followed by a rebound in circulating P4 concentrations (Carvalho et al., 2015). Our objective was to evaluate the effects of administration of 12.5 mg of PGF_{2α} (dinoprost tromethamine) 5 d after induction of the LH surge on subsequent P4 concentrations and on experimental endpoints related to embryonic growth and development. These endpoints included interferon-stimulated gene 15 (**ISG15**) mRNA expression 18 and 20 d after TAI, serum pregnancy-specific protein B (**PSPB**) concentrations from 18 to 67 d after TAI, and embryonic crown-rump length at 32, 39, and 46 d after TAI. The rationale for our experimental approach was based on results from an experiment in which we reported a negative relationship between milk production and concentrations of pregnancy-associated glycoproteins (**PAG**) in both serum and milk (Ricci et al., 2015). We speculated that cows with greater milk production may have decreased P4 concentrations during embryo development because of increased hepatic metabolism of circulating P4 (Sangsrutavong et al., 2002), which may in turn inhibit growth of the embryo, thereby resulting in decreased PAG production by the conceptus (Ricci et al., 2015). Our hypotheses were that cows treated with 12.5 mg of PGF_{2α} would have (1) temporarily decreased P4 concentrations, (2) decreased expression of ISG15 mRNA, (3) decreased serum PSPB concentrations, and (4) smaller embryos than control cows.

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.

Cows, Housing, and Feeding

This study was conducted at the University of Wisconsin–Madison Emmons Blaine Dairy Cattle Research Center located near Arlington, Wisconsin, from September to December 2015. Lactating Holstein cows ($n = 80$) were milked twice daily at approximately 12-h intervals and were fed a TMR once daily consisting

of corn and alfalfa silage as forage with corn and soybean meal-based concentrate formulated to meet or exceed the minimum nutritional requirements for high-producing dairy cows (NRC, 2001). Cows were housed in free-stall barns bedded with sand and had ad libitum access to feed and water. Primiparous cows were housed in separate pens from multiparous cows. The rolling herd average was 13,880 kg and average daily milk production was 41.6 kg/cow per d with 3.8% fat and 3.2% protein for the herd during the time period of this experiment.

Experimental Treatments

All cows were submitted to a Double-Ovsynch protocol for first TAI as described by Souza et al. (2008) and later modified by Brusveen et al. (2009) starting at 53 ± 3 DIM. The GnRH (100 µg/dose of gonadorelin hydrochloride, Factrel) and PGF_{2α} (25 mg/dose of dinoprost tromethamine, Lutalyse) were manufactured by Zoetis (Madison, NJ). Briefly, cows received the first GnRH treatment of the Pre-Ovsynch portion of the Double-Ovsynch protocol, followed by a treatment with PGF_{2α} 7 d later and treatment with GnRH 72 h after administration of PGF_{2α}. Seven days later, cows began the Breeding-Ovsynch portion of the Double Ovsynch protocol in which cows received a GnRH treatment, followed by 2 PGF_{2α} treatments administered 7 and 8 d later and the last GnRH treatment (**G2**) administered 56 h after the first PGF_{2α} treatment followed by TAI 16 to 20 h after G2. Primiparous ($n = 11$) and multiparous ($n = 69$) cows were blocked by parity (primiparous vs. multiparous) and were assigned randomly to receive no treatment (control) or to receive 12.5 mg of PGF_{2α} (**LowP4**; dinoprost tromethamine) 5 d after G2 (Figure 1). Multiple sires with high genetic merit and proven fertility were used for TAI, and sires were equally balanced between treatments.

Ultrasound Evaluation of Ovaries

Location and number of corpora lutea were evaluated weekly beginning 5 d after TAI until cows were diagnosed not pregnant or until 67 d after TAI for pregnant cows (Figure 1) by transrectal ultrasonography using a portable scanner (Ibex Pro, E. I. Medical Imaging, Loveland, CO) equipped with a 7.5-MHz linear-array transducer. Two cows lacking a corpus luteum 5 d after TAI (one control cow and one LowP4 cow) failed to synchronize in response to the Double-Ovsynch protocol and were removed from the experiment, resulting in 39 cows in each treatment.

Blood Sampling

Blood samples for P4 and PSPB were collected via puncture of the median caudal veins or arteries into 8-mL evacuated serum collection tubes (Vacutette, Greiner Bio-One North America Inc., Monroe, NC) thrice weekly beginning 5 d after G2 (i.e., immediately before treatment) until 32 d after TAI for all cows and then weekly until 67 d for cows diagnosed pregnant 32 d after TAI (Figure 1). Blood samples were refrigerated immediately after collection for 24 h and centrifuged ($1,600 \times g$; 4°C) for 20 min. Serum was harvested and stored at -20°C until assayed for P4 and PSPB. Blood samples (3 mL) for analysis of ISG15 mRNA expression were collected 4, 18, and 20 d after TAI from the coccygeal vessels into Tempus Blood RNA tubes containing 6 mL of RNA stabilization solution (Applied Biosystems, Foster City, CA). Tubes were shaken by hand vigorously for 20 s immediately after collection, and whole blood was stored at -20°C until analysis.

Analysis of Progesterone and Pregnancy-Specific Protein B

Progesterone concentrations were determined using a solid-phase, no-extraction RIA (MP Biomedicals, Solon, OH). The average sensitivity for the 3 assays was 0.027 ng/mL. The average intraassay CV was 5.6%, and the interassay CV was 6.9% based on a quality control sample (2.50 ng/mL of P4), which was replicated within each assay. Concentrations of PSPB were determined using a quantitative ELISA (Biopryn, Bio-Tracking LLC, Moscow, ID). The average intraassay CV was 5.3%, and the interassay CV was 7.4% based on a quality control sample (0.9 ng/mL of PSPB), which was replicated within each assay.

Analysis of ISG15

Total RNA was extracted from blood leukocytes using the Tempus Spin RNA Isolation Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions including the optional DNase step. Extracted RNA (1 μg) was reverse transcribed to cDNA (Bio-Rad iScript Reverse Transcription Supermix #1708840). 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 (Laporta et al., 2013). 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 (CycloA; NM_178320; forward 5'-CACCGTGTCTTCGACATCG-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). Blood samples collected 4 d after TAI served as a reference to calculate fold-change in ISG15 mRNA expression 18 and 20 d after TAI.

Pregnancy Diagnosis and Evaluation of Embryonic Crown-Rump Length

Pregnancy diagnosis and measurement of embryonic crown-rump length was performed using a portable scanner (Ibex Pro, E. I. Medical Imaging, Loveland, CO) equipped with a 7.5-MHz linear-array transducer. A positive pregnancy diagnosis was based on visualization of a corpus luteum on the ovary ipsilateral to the

Figure 1. Schematic representation of treatments and data collection. Cows were submitted to a Double-Ovsynch protocol for synchronization of ovulation for first timed artificial insemination (TAI). The first GnRH treatment of the Pre-Ovsynch portion of the Double-Ovsynch protocol was followed by treatment with $\text{PGF}_{2\alpha}$ 7 d later and GnRH 72 h after $\text{PGF}_{2\alpha}$. Seven days later, cows were treated with GnRH followed by 2 $\text{PGF}_{2\alpha}$ treatments administered 7 and 8 d later with the last GnRH (G2) treatment administered 56 h after the first $\text{PGF}_{2\alpha}$ treatment and TAI 16 to 20 h later. Five days after the last GnRH treatment, cows were assigned randomly to receive 12.5 mg of dinoprost tromethamine (LowP4) or remain untreated (control). P4 = progesterone; ISG15 = interferon stimulated gene 15; PSPB = pregnancy-specific protein B; US = transrectal ultrasonography.

uterine horn containing an embryo with a heartbeat. Pregnancy status for cows diagnosed pregnant 32 d after TAI was reconfirmed weekly until 67 d after TAI. Any cow diagnosed pregnant and then diagnosed not pregnant at a subsequent pregnancy examination was considered to have undergone pregnancy loss. Embryonic crown-rump length was measured 32, 39, and 46 d after TAI. Images were frozen when the crown-rump length was determined visually to be at its maximal size, and was then measured using the built-in digital calipers of the ultrasound machine.

Statistical Analyses

The experiment was conducted as a completely randomized design. Cows were stratified by parity (primiparous vs. multiparous) and were then randomly assigned to treatments to balance parity between treatments. All statistical analyses were performed using SAS computational software, version 9.3 for Microsoft Windows (SAS Institute Inc., Cary, NC). Analyses of binary response data (P/AI and pregnancy loss) were performed by logistic regression using the GLIMMIX procedure of SAS. The model included the fixed effects of parity and treatment. Concentrations of P4 (from 4 to 15 d, and from 18 to 67 d after TAI) and PSPB were analyzed by ANOVA using the MIXED procedure of SAS. The model contained the fixed effects of parity, treatment, time, pregnancy status, and all 2-way interactions. Due to repeated measures within each cow, a repeated statement was used to account for autocorrelations by modeling the spatial power between sequential measurements. Fold-change in ISG15 mRNA from 4 to 18 and 20 d after TAI was analyzed by ANOVA using the MIXED procedure of SAS. The model contained the fixed effects of parity, treatment, pregnancy status 32 d after TAI (pregnant vs. open), and all 2-way interactions. A significant difference between levels of a classification variable was considered when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered a statistical tendency. Data are presented as least squares means $\pm$ standard error of the mean obtained from the MIXED procedure of SAS.

RESULTS AND DISCUSSION

Several experiments have focused on the effect of increasing P4 early after ovulation on uterine physiology and early embryonic growth. Supplementation with exogenous P4 increases circulating P4 concentrations and induces changes in the endometrial transcriptome (Forde et al., 2009, 2011; Carter et al., 2010), result-

ing in a dramatic increase in embryo elongation on d 13 to 16 of pregnancy (Carter et al., 2008; Clemente et al., 2009). Progesterone exerts these effects on the embryo and conceptus indirectly by altering the uterine environment and thereby accelerating conceptus development (Clemente et al., 2009). Based on these data, supplementation with exogenous P4 after AI has been thought to be a practical strategy to increase fertility in dairy cows. Results from large field trials are equivocal, however, reporting an increase (Monteiro et al., 2014), no change (Stevenson et al., 2007; Monteiro et al., 2015), or a decrease (Parr et al., 2014) in P/AI for dairy cows treated with exogenous P4 early after ovulation compared with untreated controls.

In the present study, we took an alternative approach to assessing the effect of P4 after AI by temporarily decreasing P4 concentrations early after ovulation. Thus, our overall hypothesis was that decreasing P4 early after ovulation would decrease embryonic growth. Our experimental endpoints included various proxies for embryonic growth including ISG15 mRNA expression 18 and 20 d after TAI, serum PSPB concentrations 18 to 67 d after TAI, and embryonic crown-rump length 32, 39, and 46 d after TAI.

Effect of Treatment on Serum Progesterone Concentrations

The early bovine corpus luteum does not undergo complete luteal regression in response to a single PGF_{2 α} treatment (Rowson et al., 1972; Henricks et al., 1974; Momont and Seguin, 1984), and several studies have reported a transient decrease in circulating P4 before functional recovery of the corpus luteum (Nascimento et al., 2014; Carvalho et al., 2015). In the present experiment, we administered 12.5 mg of PGF_{2 α} 5 d after the last GnRH treatment of a Double-Ovsynch protocol to temporarily decrease P4 after TAI. Five days after G2 (i.e., immediately before administration of treatment), P4 concentrations did not differ ($P = 0.15$) between treatments (Figure 2). From 6 to 11 d after TAI, LowP4 cows had less ($P < 0.01$) serum P4 than control cows (Figure 2). After this transient decrease, serum P4 concentrations did not differ between treatments 13 ($P = 0.20$) or 15 ($P = 0.57$) d after TAI (Figure 2). Overall, P4 concentrations did not differ ($P = 0.11$) between parities (4.3 ± 0.3 vs. 3.9 ± 0.1 ng/mL for primiparous vs. multiparous cows) from 4 to 15 d after TAI.

The resulting P4 profile after administration of 12.5 mg of PGF_{2 α} in the present study (Figure 2) adds to the body of literature demonstrating that the early corpus luteum does not fully regress in response to

Figure 2. Mean ($\pm$ SEM) progesterone concentration from 4.5 to 15 d after timed artificial insemination (TAI). Cows were submitted to a Double-Ovsynch protocol for synchronization of ovulation for first TAI. Five days after the last GnRH treatment, cows were assigned randomly to receive 12.5 mg of dinoprost tromethamine (LowP4; open circles) or remain untreated (control; closed circles). Different letters (a–g) denote differences ($P < 0.05$) across time within treatment as well as between treatments.

exogenous $\text{PGF}_{2\alpha}$ (Nascimento et al., 2014). Rather, P4 transiently decreases followed by recovery of luteal function particularly when a reduced dose of $\text{PGF}_{2\alpha}$ is administered (Carvalho et al., 2015). The lack of regression of the early corpus luteum is an intriguing biological phenomenon arising from the inability of a single bolus of exogenous $\text{PGF}_{2\alpha}$ to completely activate intracellular signal transduction pathways in the early corpus luteum (Tsai and Wiltbank, 1998; Mondal et al., 2011; Diaz et al., 2013). In agreement with other studies (Mann and Lamming, 2001; Gümen et al., 2003; Sterry et al., 2009), cows diagnosed pregnant 32 d after TAI in the present study had greater mean P4 concentrations from 4 to 15 d after TAI than cows diagnosed not pregnant (4.6 ± 0.2 vs. 3.7 ± 0.2 ng/mL, respectively).

From 15 d after TAI onward, P4 concentrations did not differ ($P = 0.82$) between treatments (6.6 ± 0.2 vs. 6.7 ± 0.2 ng/mL, for control vs. LowP4 cows) or ($P = 0.82$) parities (7.3 ± 0.4 vs. 6.6 ± 0.2 ng/mL, for primiparous vs. multiparous cows). Overall P4 concentrations for cows that became pregnant remained high from 18 to 67 d after TAI, whereas P4 concentrations for nonpregnant cows began to decrease 18 d after TAI (Figure 3). Cows identified not pregnant 32 d after TAI in the present study were resynchronized for second TAI and blood sampling was discontinued. No treatment by pregnancy status interaction ($P = 0.87$) was present for P4 from 18 to 67 d after TAI.

Effect of Treatment on ISG15 mRNA Expression

Interferon-tau is a member of the type I IFN family (Roberts et al., 1997) and is an early signal for maternal recognition of pregnancy in ruminants (Spencer and Bazer, 2004). Interferon-tau is secreted by the mononuclear trophoblast cells of the ruminant conceptus (reviewed by Spencer et al., 2007, and Bazer et al., 2012), which in turn induces expression of a large number of IFN-stimulated genes in the uterine endometrium (Gray et al., 2006) and in circulating blood leukocytes (Gifford et al., 2007; Green et al., 2010). Several IFN-stimulated genes have been used to determine pregnancy status in dairy cows including ISG15 (Han et al., 2006; Gifford et al., 2007).

In the present experiment, we assessed the effect of both treatment and pregnancy status on fold-change in ISG15 mRNA expression from 4 to 18 and 20 d after TAI. At 18 d after TAI, pregnant cows had a 4-fold increase ($P < 0.01$) in ISG15 relative mRNA expression compared with 4 d after TAI than nonpregnant cows (Figure 4); however, relative mRNA expression did not differ between treatments ($P = 0.78$) or parities ($P = 0.21$), and no treatment by pregnancy status interaction ($P = 0.64$) was present. At 20 d after TAI, pregnant cows had a 6-fold increase ($P < 0.01$) in ISG15 mRNA expression relative to 4 d after TAI compared with nonpregnant cows (Figure 4). Relative ISG15 mRNA expression did not differ ($P = 0.64$) between treatments for nonpregnant cows 20 d after TAI; however, pregnant control cows had 3-fold greater ($P = 0.04$) relative ISG15 mRNA expression relative to 4 d after TAI than pregnant LowP4 cows (Figure 4).

Figure 3. Mean ($\pm$ SEM) progesterone concentration from 18 to 67 d after timed artificial insemination (TAI) for cows diagnosed pregnant (closed circles) or not pregnant (open circles) 32 d after TAI.

Figure 4. Mean ($\pm$ SEM) relative fold-change in interferon-stimulated gene 15 (ISG15) from 4 to 18 and 20 d after timed artificial insemination (TAI). Cows were submitted to a Double-Ovsynch protocol for synchronization of ovulation for first TAI. Five days after the last GnRH treatment, cows were assigned randomly to receive 12.5 mg of dinoprost tromethamine (LowP4; open bars) or remain untreated (control; shaded bars). Different letters (a–c) within a day denote differences ($P < 0.05$) between treatment and pregnancy status.

Concentrations of IFN-tau in uterine flushings have been correlated with trophoblast length (Mann et al., 2006) and the amount of mRNA for IFN-stimulated genes isolated from peripheral blood leukocytes (Matsuyama et al., 2012). Therefore, increased ISG15 mRNA expression 16 d after AI in cows with high circulating P4 after AI (Forde et al., 2009), and decreased ISG15 mRNA expression 20 d after TAI in cows with low P4 in the present study (Figure 4), support the idea that decreasing circulating P4 concentrations early after ovulation decreased embryonic growth. These data are interesting in light of another study conducted in Irish Holstein-Friesian cows in which cows manipulated to have low circulating P4 during growth of the preovulatory follicle before TAI had increased levels of ISG15 mRNA expression in blood leukocytes 18 d after TAI compared with cows manipulated to have high circulating P4 before TAI (Fricke et al., 2016). The day after TAI that ISG15 mRNA expression was assessed (i.e., 18 vs. 20) and the physiology of high-producing dairy cows used in the present experiment versus moderately producing Irish-Holstein Friesian cows managed in a pasture-based system may account for these differences in ISG15 mRNA expression.

Effect of Treatment on Serum PSPB Concentrations

In 1982, 2 proteins, pregnancy-specific proteins A and B, were isolated from bovine fetal membrane extracts

(Butler et al., 1982). Development of a specific RIA for PSPB allowed for quantification of PSPB in maternal serum as an indirect method for pregnancy diagnosis (Sasser et al., 1986) and assessment of pregnancy loss (Giordano et al., 2012) in dairy cows. Molecular cloning and sequencing studies revealed that PAG belong to a large family of inactive aspartic proteinases expressed by the placenta of domestic ruminants including cows, ewes, and goats (Haugejorden et al., 2006). We (Ricci et al., 2015) and others (López-Gatius et al., 2007) have reported a negative relationship between milk production and concentrations of circulating PAG. We speculated that cows with greater milk production may have decreased P4 concentrations because of increased hepatic metabolism of circulating P4 (Sangsritavong et al., 2002), which may in turn inhibit growth of the embryo, resulting in decreased PAG production by the embryo (Ricci et al., 2015). This was our primary rationale for assessing the effect of temporarily decreasing P4 early after TAI on embryonic growth in the present experiment.

Overall, serum PSPB concentrations for cows diagnosed pregnant 32 d after TAI in the present experiment were low 18 and 20 d after TAI for cows in both treatments and then began to increase 22 d after TAI (Figure 5). Similar to serum PSPB (Sasser et al., 1986) and PAG profiles in serum and milk (Ricci et al., 2015) reported in other studies, overall serum PAG concentrations increased to an early peak 32 d after TAI, then transiently decreased from 32 to 46 d after TAI followed by an increase from 53 to 67 d after TAI. Control cows had greater ($P = 0.04$) serum PSPB concentrations than LowP4 cows from 25 to 67 d after TAI (Figure 5). No treatment by time interaction ($P = 0.29$) was present. In contrast to other studies that measured serum PAG (Ricci et al., 2015; Fricke et al., 2016) and milk PAG (Ricci et al., 2015) in lactating Holstein cows, concentrations of PSPB did not differ ($P = 0.22$) between parities (1.5 ± 0.1 vs. 1.3 ± 0.1 ng/mL, for primiparous vs. multiparous cows).

Data from PSPB concentrations in the present study further support the idea that temporarily decreasing P4 after TAI decreased embryonic growth. Whereas the effect of treatment on P4 after AI was temporary and restricted to early pregnancy (i.e., from 6 to 11 d after TAI; Figure 2), the effect of treatment on serum PSPB concentrations was manifested 25 to 67 d after TAI (Figure 5), suggesting a long-term effect of treatment on embryonic and fetal growth. Further, the pattern of decreased PSPB in Figure 5 is not only due to a delay in embryonic and fetal growth because PSPB levels for lowP4 cows never reached PSPB concentrations of control cows (i.e., the PSPB pattern over time

for LowP4 cows was not simply shifted to the right). Data from Figure 5 indicate that although decreased P4 early after TAI decreased overall PSPB concentrations, factors other than embryonic size account for the temporal change in PSPB during early pregnancy (i.e., the transient dip in PSPB concentrations occurring from 32 to 67 d after TAI). This temporal pattern in PSPB and PAG concentrations has been consistently reported among studies and remains an intriguing biological phenomenon that is not completely understood.

Effect of Treatment on Embryonic Crown-Rump Length

Crown-rump length of embryos and fetuses recovered from dairy cows of known gestational age is highly correlated with stage of pregnancy (Rexroad et al., 1974), and transrectal ultrasonography is a noninvasive and highly accurate method for measuring embryonic crown-rump length in dairy cows (Hughes and Davies, 1989). In the present study, embryonic crown-rump length increased ($P < 0.01$) from 32 to 46 d after TAI for cows in both treatments (Table 1). At 32 d after TAI, embryonic crown-rump length did not differ ($P = 0.84$) between treatments (Table 1); however, primiparous cows had smaller ($P = 0.02$) embryos than multiparous cows (10.1 ± 1.3 vs. 12.4 ± 0.6 mm, respectively). At 39 d after TAI, embryonic crown-rump length did not differ ($P = 0.59$) between treatments (Table 1) or parities ($P = 0.20$; 14.5 ± 1.3 vs. 16.6

Table 1. Effect of treatment (mean $\pm$ SEM) on embryonic crown-rump length (mm) 32, 39, and 46 d after timed artificial insemination (TAI)

Days after TAI	Treatment ¹		<i>P</i> -value
	Control	LowP4	
32	10.8 ± 1.0	11.6 ± 0.8	0.84
39	15.0 ± 1.0	16.1 ± 0.8	0.59
46	26.3 ± 1.0	24.0 ± 0.8	0.02

¹Cows were submitted to a Double-Ovsynch protocol for synchronization of ovulation to receive first TAI. Five days after the last GnRH treatment, cows were assigned randomly to receive 12.5 mg of dinoprost tromethamine (LowP4) or remain untreated (control).

± 0.6 mm for primiparous vs. multiparous cows). In contrast, embryonic crown-rump length was greater ($P = 0.02$) for control than for LowP4 cows 46 d after TAI, but did not differ ($P = 0.62$) between parities (25.9 ± 1.3 vs. 24.4 ± 0.6 mm for primiparous vs. multiparous cows).

Data on crown-rump length 46 d after TAI in the present study directly supports that decreasing P4 early after TAI decreased embryonic growth. Similar to the effect of treatment on serum PSPB, the effect of treatment on embryonic crown-rump length was manifested long after the transient decrease in P4 after TAI occurred. Although we do not know why embryonic crown-rump length did not differ 32 or 39 d after TAI, we speculate that small differences in crown-rump length at these early stages of embryonic development may have exceeded the sensitivity of transrectal ultrasonography to detect them.

Effect of Treatment on P/AI and Pregnancy Loss

Overall, P/AI did not differ between treatments, and 43.6% (17/39) of cows in each treatment were diagnosed pregnant 32 d after TAI (Table 2). At 32 d after TAI, P/AI did not differ ($P = 0.44$) between parities [54.5% (6/11) vs. 41.8% (28/67) for primiparous vs. multiparous cows]. Pregnancy loss between 32 and 67 d after TAI did not differ ($P = 0.79$) between treatments (Table 2) or ($P = 0.30$) parities [16.7% (1/6) vs. 3.6% (1/28) for primiparous vs. multiparous cows]. Although this experiment was not statistically powered to detect treatment effects on P/AI or pregnancy loss, the proportion of pregnant cows in each treatment was identical 32, 39, 53, 60, and 67 d after TAI, and only one cow in each treatment underwent pregnancy loss from 32 to 67 d after TAI (Table 2). Thus, decreasing P4 early after TAI did not have a lethal effect on the embryo. Further, these proportions of primiparous and multiparous cows diagnosed pregnant 32 d after TAI are typical for cows submitted to TAI after the Double-

Figure 5. Mean ($\pm$ SEM) pregnancy-specific protein B (PSPB) concentrations for pregnant cows from 18 to 67 d after timed artificial insemination (TAI). Cows were submitted to a Double-Ovsynch protocol for synchronization of ovulation for first TAI. Five days after the last GnRH treatment, cows were assigned randomly to receive 12.5 mg of dinoprost tromethamine (LowP4; open circles) or remain untreated (control; closed circles).

Table 2. Effect of treatment on pregnancies per artificial insemination (P/AI) from 32 to 67 d after timed artificial insemination (TAI) and pregnancy loss from 32 to 67 d after TAI

Item [% (no./no.)]	Treatment ¹		P-value
	Control	LowP4	
Days after TAI			
32	43.6 (17/39)	43.6 (17/39)	0.98
39	43.6 (17/39)	43.6 (17/39)	0.98
46	41.0 (16/39)	43.6 (17/39)	0.84
53	41.0 (16/39)	41.0 (16/39)	0.99
60	41.0 (16/39)	41.0 (16/39)	0.99
67	41.0 (16/39)	41.0 (16/39)	0.99
Pregnancy loss	5.9 (1/17)	5.9 (1/17)	0.79

¹Cows were submitted to a Double-Ovsynch protocol for synchronization of ovulation to receive first TAI. Five days after the last GnRH treatment, cows were assigned randomly to receive 12.5 mg of dinoprost tromethamine (LowP4) or remain untreated (control).

Ovsynch protocol used for synchronization of ovulation in the present experiment.

The present experiment showed no indication that decreasing P4 from 6 to 11 d after TAI had a major effect on fertility or pregnancy loss, bringing into question the idea that increasing P4 early after AI might be a practical strategy to increase fertility in lactating dairy cows. During the normal estrous cycle, downregulation of endometrial P4 receptors is followed by appearance of estrogen receptor α and oxytocin receptors leading to the pulsatile secretion of PGF_{2 α} , which induces luteolysis (McCracken et al., 1999). Progesterone downregulates its own receptor, and loss of endometrial P4 receptors seems to be required for secretion of histotroph (Spencer and Bazer, 2002) and implantation (Spencer and Bazer, 1995; Lessey et al., 1996; Kimmins and Maclaren, 2001). Interestingly, nuclear P4 receptor expression was greater in heifers with low P4 concentrations 7 d after ovulation than control heifers (Forde et al., 2012). In addition, supplementation with exogenous P4 from 4 to 18 d after AI increased the percentage of cows undergoing luteolysis by 19 d after AI (Monteiro et al., 2014). Thus, although increasing P4 concentrations after AI advanced embryo elongation, early downregulation of endometrial P4 receptors and advancement of the luteolytic cascade mechanism could explain why increasing P4 concentrations after AI has equivocal effects on P/AI among studies. In this regard, recent studies reported minimal or no benefit of increasing P4 concentrations after AI on P/AI in dairy cows (Nascimento et al., 2013; Yan et al., 2016).

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