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Effects of preovulatory estradiol on embryo survival and pregnancy establishment in beef cows☆

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ABSTRACT

The role of preovulatory estradiol on post-fertilization embryo survival and pregnancy establishment has not been well characterized in beef cows. We hypothesized that preovulatory estradiol is important for embryo survival and pregnancy establishment in beef cows. Twenty-four ovariectomized multiparous cows were used in a replicated 3 × 3 Latin Square design. Cows received estradiol cypionate (ECP) 36 h, estradiol benzoate (EB) 12 h, or no estradiol (CON) before a gonadotropin-releasing hormone (d 0) induced LH surge. Luteal phase progesterone was mimicked with twice daily progesterone injections from d 3 to 6. On d 7 cows received one embryo and progesterone was supplemented with progesterone-releasing devices (CIDR). Expression of interferon stimulated genes, *ISG15*, *MX2*, and *OAS1*, in leukocytes was determined on d 17, 19, 21, and 28 to determine capability of embryonic signaling. Pregnancy specific protein B concentrations were measured in serum samples from d 17 through 29 to determine embryonic attachment. Transrectal ultrasonography was performed on d 29 and 32 to determine pregnancy viability (heartbeat). Serum estradiol profiles during simulated proestrus/estrus were different ($P < 0.001$) between treatments. Mean serum progesterone concentrations from d 17 to 24 were decreased ($P = 0.05$) in EB and ECP cows compared to CON. Transrectal ultrasonography indicated that fewer CON (4%) cows had a viable embryo present compared to estradiol treated cows (25%). Embryonic loss in cows that did not receive estradiol during the simulated preovulatory period occurred following maternal recognition of pregnancy, indicating that its impact was likely on uterine receptivity and embryonic attachment.

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1. Introduction

The role of estradiol during the preovulatory period on post-fertilization embryo survival and pregnancy establishment has not been well characterized in beef cows. Serum estradiol concentrations have been positively correlated with ovulatory follicle size (Perry et al., 2005; Atkins et al., 2010) and cows ovulating smaller follicles following gonadotropin-releasing hormone (GnRH) injection experienced greater reproductive failure (Perry et al., 2005; Jinks et al., 2013). Bridges et al. (2010) reported greater serum estradiol concentrations and pregnancy rates among cows induced to ovulate a follicle of similar size but allowed a longer period of proestrus. Decreased levels of estradiol in the preovulatory period have also been associated with premature luteolysis (Mann and Lamming, 2000). While increased preovulatory estradiol is essential for optimum fertility, earlier studies have not allowed separation of the direct effects of estradiol from other fertility traits (i.e. sperm transport, fertilization, subsequent concentrations of progesterone, etc.).

Miller et al. (1977) evaluated the effects of estradiol in ovariectomized ewes by treatment with low estradiol (single injection of 25 μg) or high estradiol (35 μg distributed across 5 injections) to mimic the preovulatory period. Following embryo transfer on d 4, ewes that did not receive the elevated estradiol supplementation were less likely to maintain a normal embryo, had reduced uterine weight on d 21, and had reduced amounts of uterine luminal proteins on d 21 (Miller et al., 1977). The above body of work suggests a direct effect of estradiol on embryo development and survival. Several estrous and ovulation synchronization studies have been conducted using estradiol cypionate or estradiol benzoate treatments during proestrus with reported benefits in pregnancy rate (Ahmadzadeh et al., 2003; Colazo et al., 2004). Souza et al. (2005) demonstrated differential estradiol profiles in cattle using estradiol benzoate or estradiol cypionate. We hypothesized that cows exposed to estradiol during the preovulatory period would have increased embryonic survival and pregnancy establishment when all other factors were held constant. In order to test the effects of estradiol exposure during the preovulatory period on embryo survival, two forms of estradiol were utilized: estradiol cypionate (ECP) and estradiol benzoate (EB). The objective of this study was to examine the role of preovulatory estradiol on survival of embryos transferred on d 7, and pregnancy establishment without the effects of ovulatory follicle size, endogenous estradiol secretion, proestrous interval, or subsequent progesterone exposure.

2. Materials and methods

2.1. Ethics

All procedures involving animals used in this research were approved by the Fort Keogh Livestock and Range Research Laboratory Animal Care and Use Committee and the South Dakota State University Animal Care and Use Committee.

2.2. Animals and treatments

Crossbred multiparous beef cows ($n = 24$; 579 ± 58 kg body weight) were ovariectomized by lateral lumbar incision on d 0 of the estrous cycle and randomly assigned to a 3×3 Latin Square experimental design with 8 replicates per phase. Immediately after surgery, cows received a progesterone-releasing device (CIDR; Eazi-Breed CIDR containing 1.38 g of progesterone, Pfizer Animal Health) that was changed weekly for 21 d to mimic a luteal phase. The experiment was performed in 3 phases with the first 2 phases at Fort Keogh and the final phase at the South Dakota State University Beef Breeding Unit (8 animals per treatment per phase; $n = 24$ animals per treatment total). Phases were separated by approximately five weeks from the end of one phase to the start of synchrony for the next phase. Nine days before simulation of the onset of estrus (d 0) cows received a CIDR. On d −2, CIDRs were removed and cows were given a single injection of dinoprost tromethamine (PGF2α; 25 mg, i.m.). On d 0 (48 h post PGF2α) gonadotropin-releasing hormone injection (GnRH; 100 μg, i.m.) was administered to stimulate the LH surge that occurs at the onset of estrus. According to the Latin Square assignment, cows were assigned to one of three treatments for each phase of the experiment: ECP (2.5 mg [1 mg/ml in sesame oil carrier, i.m.] 36 h prior to GnRH), EB (1.2 mg [1 mg/ml in sesame oil carrier, i.m.] 12 h prior to GnRH), or CON (no exogenous estradiol). Prior to GnRH, all animals in the EB or ECP treatment exhibited estrus, as determined by activation of estrus-detecting patches (Estrotect, Rockway Inc.). On d 3, 4, 5, and 6, all cows received injectable progesterone (10 mg/ml in sesame oil carrier, i.m.) in the following doses: 40, 80, 160, and 240 mg, respectively. Daily progesterone doses were dived into two injections given 12 h apart. On d 7, all cows received a single embryo (stage 4, 5, or 6; quality grade 1 or 2; all stages and grades were equally assigned across treatments) and a single (500 mg, i.m.) injection of flunixin meglumine (Merck Animal Health), and a CIDR. The embryo transfer technician was the same throughout the entire study. On d 8, cows received an additional CIDR, and every 6 d thereafter through d 29, the older of the two CIDRs was removed and replaced with a new CIDR.

2.3. Ultrasonography

Pregnancy status for each animal was determined by transrectal ultrasonography using an Aloka SSD-3500 Ultrasound with a 10 MHz convex probe or an Aloka 500 with a 7.5 MHz linear probe (Aloka) on d 29 and confirmed on d 32. Ultrasound technician was blind to treatment. Viability of an embryo was confirmed by presence of a heartbeat.

2.4. Blood collection and RIA

Blood was collected by coccygeal or jugular venipuncture into 10 ml vacutainer tubes (BD Vacutainer) at the following times relative to GnRH: d −9, d −2, every 4 h from −36 h to 0 h, every 30 min from GnRH until h 2.5, d 3, 4, 5, 6, 7, and d 14. Samples were placed at 4 °C for approximately

24 h. Samples were centrifuged at 1200 × *g* for 25 min at 4 °C and serum was collected and stored at −20 °C until radioimmunoassays were performed. Radioimmunoassay (RIA) was performed on serum samples to measure progesterone concentrations using the methods described by Engel et al. (2008). Inter- and intra-assay CV were 13.67% and 4.52%, respectively and assay sensitivity was 0.4 ng/mL. In order to characterize circulating estradiol profiles from −48 h until GnRH administration, RIA was performed using the methods described by Perry and Perry (2008). Inter- and intra-assay CV were 18.1% and 5.7%, respectively and assay sensitivity was 0.5 pg/mL. To determine the LH response to the GnRH injection, serum samples collected from −36 h to 2.5 h after the GnRH injection were analyzed using RIA according to methods described by Perry and Perry (2008). Inter- and intra-assay CV were 13.0% and 4.0%, respectively and assay sensitivity was 01.25 ng/mL.

2.5. Collection of blood leukocytes and RT-PCR

Plasma and blood leukocytes were collected by jugular venipuncture into 10 ml vacutainer tubes containing EDTA (BD Vacutainer) on d 17 through 28 relative to GnRH. The blood was placed on ice immediately after collection and centrifuged at 1200 × *g* for 25 min at 4 °C within 1 h of collection. Plasma was collected and stored at −20 °C until used for RIA (progesterone, described above) or pregnancy specific protein B (PSPB) analysis. Blood leukocytes were collected, mixed in a 1:1 volumetric ratio with Tri-Reagent (Molecular Research Center, Inc.), and stored at −80 °C until RNA isolation. Isolation of RNA was performed using an SV Total RNA Isolation System (Promega) according to manufacturer's instructions. Nuclease free water was used to dilute DNase treated RNA and concentration was determined using a spectrophotometer (NanoDrop Technologies). Isolated RNA samples were stored at −80 °C. The RNA collected on d 17, d 19, d 21, and d 28 were diluted to 12 ng/μl and RT-PCR was performed in triplicate using iScript One-Step RT-PCR Kit with SYBR Green (BioRad). Expression of *ISG15*, *MX2*, *OAS1*, and *GAPDH* was measured using the primers listed in Table 1. Amplification occurred using a Stratagene MX3000P (d 19 samples) or an ABI Prism 7000 (d 17, d 21 and d 28 samples). The housekeeping gene *GAPDH* was used as an internal control and each plate contained negative controls to detect any background contamination. The SYBR Green reaction was performed for genes with the reverse transcription at 42 °C for 30 min and 95 °C for 10 min to inactivate reverse transcription. For each of the genes of interest, transcription was followed by 40 cycles of 30 s at 95 °C for melting; 1 min at 60 °C for annealing; and 1 min at 72 °C for extension. Primers (Table 1) were previously published for *GAPDH* (Han et al., 2006), *ISG15*, *MX2*, and *OAS1* Green et al., 2010). Amplicons were confirmed for product size on 2% agarose gels and were verified for identity by sequencing (Iowa State University Genomics Core).

2.6. Pregnancy specific protein B (PSPB) analysis

Plasma collected on d 17 through 28 was analyzed for the presence and concentration of PSPB. Serum collected on d 7 at embryo transfer was included as a negative control or baseline sample. Pregnancy-specific protein B concentrations were measured in duplicate in plasma or serum by BioTracking, LLC (BioPRYN ELISA). Inter- and intra-assay CV were each less than 10% and sensitivity of the assay was 0.1 ng/ml.

2.7. Statistical analysis

The experiment was designed as a series of nine 3 × 3 Latin squares. One square was eliminated from the analysis due to removal of a cow with uterine adhesions to the abdominal wall that was detected during the first phase of study. Analyses of hormone and individual gene expression profiles employed mixed linear models methodology, and when the binary variable, pregnancy, was the dependent variable, logistic regression was used. Hormone profiles and expression profiles were analyzed as repeated measures using split-plot in time models. The whole plot was a randomized complete block design with squares as blocks and treatments, cow, and phase were factors included in the Latin squares. In the initial analyses of expression profiles, a linear covariate was also included in the model to adjust for expression of the housekeeping gene *GAPDH*. Whole plot error was a composite of the cow × period × treatment interaction mean square (the usual error for a single Latin square) pooled over squares and pooled interactions of square with the factors included in the Latin squares (the usual error for a randomized complete block design). Note, when the pooled Latin square error means square was tested against the randomized complete block error mean square, it did not approach significance ($P > 0.20$). The sub-plot consisted of hours relative to GnRH and two-factor interactions of time with each factor included in the Latin squares. Residual variance was considered error for effects in the subplot. The analysis of pregnancy did not include the subplot, but was

Table 1
Genes, primer sequences, and primer locations for genes amplified during RT-PCR.

Gene	Primer	Primer sequence	Primer location	Reference
ISG15	Forward	5′-CAGCCAACCAGTGTCTGCAGAGA-3′	14–36	Green et al. (2010)
	Reverse	5′-CCAGGATGGAGATGCAGTTCTGC-3′	284–306	
MX2	Forward	5′-CTTCAGAGACGCCTCAGTCG-3′	2071–2090	Green et al. (2010)
	Reverse	5′-TGAAGCAGCCAGGAATAGTG-3′	2283–2302	
OAS1	Forward	5′-ACCCTCTCCAGGAATCCAGT-3′	1157–1176	Green et al. (2010)
	Reverse	5′-GATTCTGGTCCCAGGTCTGA-3′	1336–1355	
GAPDH	Forward	5′-GATTGTCAGCAATGCCTCCT-3′	543–562	Han et al. (2006)
	Reverse	5′-GGTCATAAGTCCCTCCACGA-3′	617–636	

otherwise similar to the analyses described above. Effects of gene expression (adjusted for expression of GAP) on pregnancy rate were also assessed using logistic regression.

Expression of ISG15, Mx2, and OAS1 on days 17, 19, 21, and 28 was further analyzed using the general linear model for the Latin square assignment (treatment, cow, and phase) PCR plate assignment, and *GAPDH* expression. Residuals generated for each animal were then used in discriminate analyses for d 17, 19, 21, and 28 with d 29 ultrasound pregnancy diagnoses as the class variable to generate a binary pregnancy prediction of gene expression (GE) for each of the 4 d measured (GEd17, GEd19, GEd21, GEd28). The residuals for each of the 3 genes of interest measured on d 17, 19, and 21 were also pooled in a single discriminate analysis, with d 29 ultrasound pregnancy outcome as the class variable, which generated a single binary pregnancy prediction for d 17, 19, and 21 ISG expression (GEPOOL).

Pregnancy specific protein B results for d 22–28 were analyzed using the general linear model and standardized for Latin square assignment, cow, phase, and treatment. Initially, d 28 PSPB residuals were analyzed in a discriminate analysis with d29US as the class variable. This generated a binary pregnancy prediction for d 28 PSPB (pregd28PSPB). This binary pregnancy prediction factor was then used as the class variable in a discriminate analysis for each of the PSPB measurements d 22–28. Pregnancy predictions from d 24 through d 28 were then combined in a single discriminate analysis to generate a pooled PSPB pregnancy prediction by discriminate analysis with pregd28PSPB as class variable. Concentrations of PSPB from d 24 to d 28 in cows determined to be pregnant either by pregd28PSPB or d 29 ultrasound were also analyzed as repeated measures.

Categorical data modeling with maximum likelihood estimation of parameters for log-linear models was utilized to compare predicted pregnancy rates between treatments for each of d 17, 19, and 21 through 29 as well as predicted pregnancy rate by GEPOOL and pooled PSPB pregnancy predictions. Both estradiol treatments, EB and ECP, were combined and pregnancy rates and losses of cows receiving estradiol in the preovulatory period were compared with cows in the CON treatment.

3. Results

3.1. Hormone profiles

There was a treatment, time, and treatment by time interaction ($P < 0.01$) on serum estradiol concentrations during the simulated preovulatory period, with circulating concentrations of estradiol being greater in both EB and ECP cows than CON cows (Fig. 1). No cows in the CON treatment exhibited estrus behavior. However, all cows within the EB and ECP treatments exhibited estrus behavior between d −1 and d 0. There was no treatment ($P = 0.14$) effect on circulating concentrations of progesterone, but there was an effect of time ($P < 0.01$) and a treatment by time interaction ($P = 0.05$) with serum progesterone concentrations decreased in EB and ECP cows compared to the CON cows during the plateau phase (d 19–22). However, there was no difference between EB and ECP cows (Fig. 2). There was an effect of treatment on peak serum concentration of LH with EB treated cows having greater ($P < 0.01$) peak LH concentrations (20.5 ± 3.0 ng/mL) than ECP (9.9 ± 3.0 ng/mL) or CON (10.6 ± 3.1 ng/mL). In addition, interval from GnRH to peak LH concentration differed ($P < 0.01$) with ECP treated cows reaching peak LH concentrations 638.3 ± 41.8 min before GnRH and EB and CON cows reaching peak LH concentrations after GnRH (83.3 ± 41.8 and 35.3 ± 43.0 min, respectively). There was an effect of treatment, time, and a treatment by time interaction ($P < 0.01$) on circulating concentrations of LH, with ECP treated cows having increased concentrations of LH at hour −16, −12, −8, and −4 compared to EB and CON. However, EB had greater concentrations of LH than ECP at 30, 60, 90, 120, and 150 min after GnRH and CON having greater concentrations of LH at 30 and 60 min after GnRH compared to ECP (Fig. 3).

3.2. Interferon stimulated gene expression

Expression of *ISG15*, *MX2*, and *OAS1* on d 17, 19, 21, and 28 after simulated ovulation were not different between treatments ($P > 0.10$). The expression of these three genes on each of the days measured was greater ($P < 0.01$) in cows that were confirmed to be pregnant with ultrasonography when compared to cows that were not pregnant. Expression of *ISG15* and *MX2* differed ($P < 0.01$) due to day, but expression of *OAS1* did not differ ($P > 0.10$) by day.

Expression of each of the three ISGs measured on d 17, 19, 21, and 28 was used in a combined analysis to generate binary pregnancy prediction factors for each cow on d 17, 19, 21, pooled prediction from d 17–21 combined, and d 28 (GEd17, GEd19, GEd21, GEPOOL, GEd28, respectively; Table 2). Treatment with estradiol had no effect ($P > 0.10$) on the pregnancy prediction generated by combined expression of *ISG15*, *MX2*, and *OAS1* on d 17, 19, 21 and 28.

3.3. Pregnancy-specific protein B

Concentration of PSPB was measured in blood samples collected on d 7 and 17–28, but was only consistently detected among samples collected on d 22–28 (Table 2). Presence of PSPB in the plasma on d 24 correctly predicted pregnancy in 10 of the 14 cows (71%) identified as pregnant by PSPB presence on d 28 and 10 of the 13 cows (77%) identified as pregnant by ultrasound on d 29; indicating limited usefulness for d 24 PSPB for pregnancy detection in this study. Presence of PSPB on d 28, however, identified 11 of the 13 cows identified on d 29 as pregnant as well as three seemingly "false positive" females found to be non-pregnant on d 29 by ultrasound, however, embryonic mortality was suspected by the ultrasound technician in two of the three due to presence of fluid and membranes, but no conceptus. It is possible that embryonic mortality occurred in the third false positive female and was not detectable by ultrasound on d 29. Thus, pregnancy status by d 28 PSPB or d 29 ultrasound were both considered useful indicators of pregnancy maintenance. Cows that received estradiol in the preovulatory period were more likely ($P = 0.05$) to have a positive pregnancy

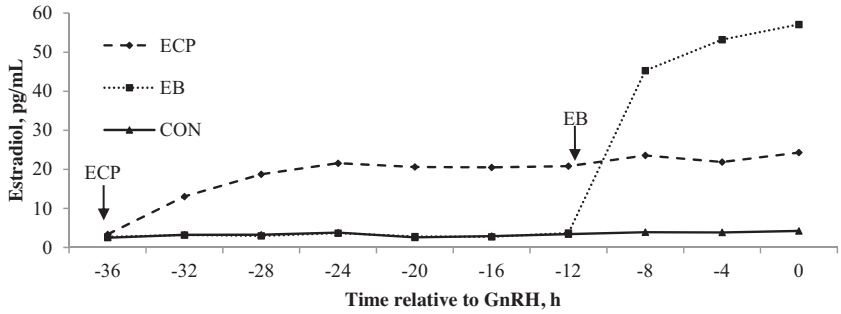

Fig. 1. Serum estradiol concentration of ovariectomized cows that received ECP, EB, or CON treatment during the simulated preovulatory period. Cows treated with ECP received 2.5 mg 36 h prior to GnRH (0 h), cows treated with EB received 1.2 mg 12 h prior to GnRH, and CON cows received no exogenous estradiol. There was a treatment, time, and treatment by time interaction ($P < 0.01$) on serum estradiol concentrations during the simulated preovulatory period.

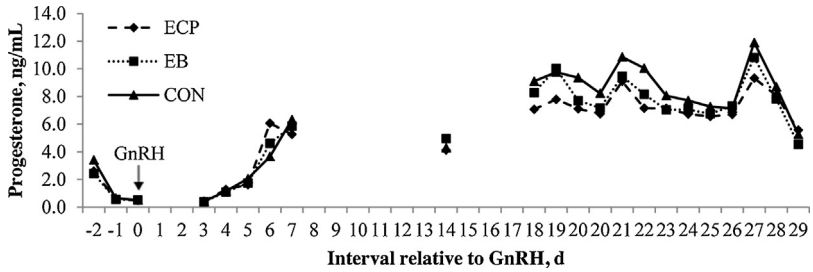

Fig. 2. Serum progesterone concentration of ovariectomized cows that received ECP, EB, or CON treatment during the simulated preovulatory period. Cows treated with ECP received 2.5 mg 36 h prior to GnRH (d 0), cows treated with EB received 1.2 mg 12 h prior to GnRH, and CON cows received no exogenous estradiol. There was no treatment ($P = 0.14$) effect on circulating concentrations of progesterone, but there was an effect of time ($P < 0.01$) and a treatment by time interaction ($P = 0.05$) with serum progesterone concentrations decreased in EB and ECP cows compared to the CON cows during the plateau phase (d 19–22). However, there was no difference between EB and ECP cows.

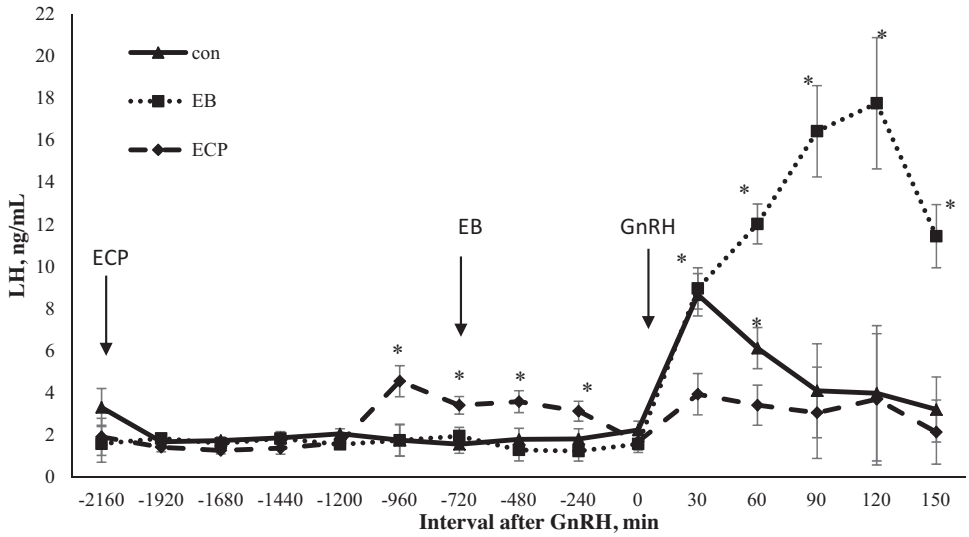

Fig. 3. Serum luteinizing hormone concentration of ovariectomized cows that received ECP, EB, or CON treatment during the simulated preovulatory period. Cows treated with ECP received 2.5 mg 36 h prior to GnRH (d 0), cows treated with EB received 1.2 mg 12 h prior to GnRH, and CON cows received no exogenous estradiol. There was an effect of treatment, time, and a treatment by time interaction ($P < 0.01$) on circulating concentrations of LH, with ECP treated cows having increased concentrations of LH at hour −16, −12, −8, and −4 compared to EB and CON. However, EB had greater concentrations of LH than ECP at 30, 60, 90, 120, and 150 min after GnRH and CON having greater concentrations of LH at 30 and 60 min after GnRH compared to ECP (*$P < 0.05$).

Table 2
Pregnancy rates as predicted by discriminate analysis across treatments on d 7, 17, 19, and 21–29.

	Day of study	ECP[a]	EB[b]	CON[c]	*P*-value	E vs. CON*P*-value[1]
ISG expression	7	100%	100%	100%	N/A	N/A
	17	33.3%	41.7%	29.2%	0.65	0.47
	19	25.0%	37.5%	25.0%	0.56	0.57
	21	41.7%	37.5%	29.2%	0.64	0.37
	28	41.7%	37.5%	33.3%	0.84	0.60
	GE Pooled	33.3%	37.5%	33.3%	0.94	0.86
PSPB concentration	22	16.7%	16.7%	8.3%	0.56	0.29
	23	20.8%	20.8%	8.3%	0.31	0.12
	24	25.0%[w]	25.0%[w]	8.3%[x]	0.14	0.05
	25	25.0%	20.8%	12.5%	0.50	0.25
	26	25.0%	20.8%	12.5%	0.50	0.25
	27	20.8%	25.0%	8.3%	0.20	0.08
	28	25.0%	20.8%	12.5%	0.50	.25
	PSPB Pooled	20.8%	25.0%	8.3%	0.20	0.08
	29	20.8%[y]	29.2%[y]	4.2%[z]	0.02	0.005

[a] 2.5 mg estradiol cypionate 36 h prior to GnRH on d 0.
[b] 1.2 mg estradiol benzoate 12 h prior to GnRH on d 0.
[c] No exogenous estradiol.
[wx] Within a row having different superscripts tended to differ ($P < 0.20$).
[yz] Within a row having different superscripts are different ($P < 0.05$).
[1] E vs. CON *P*-value compares cows that received either the EB or the ECP treatment with cows receiving the CON treatment.

prediction from the d 24 PSPB concentration compared to CON treated cows.

3.4. Ultrasonography

Transrectal ultrasonography on d 29 revealed pregnancy rates of 21%, 29%, and 4% for the ECP, EB, and CON treatments, respectively. When the two estradiol treatments were combined and compared to the CON treatment, cows receiving estradiol in the mimicked preovulatory period were more likely ($P = 0.005$) to maintain pregnancy to d 29 (Fig. 4).

3.5. Pregnancy loss

Extensive blood sampling and the subsequent measurements of ISG expression, PSPB concentration, and ultrasound generated a total of 13 pregnancy prediction outcomes for each animal over the course of d 17 to d 29. Fig. 4 illustrates the cumulative loss of pregnancy and the stages of pregnancy loss from embryo transfer on d 7 to ultrasonography on d 29. It further shows no difference ($P = 0.86$) in pregnancy loss rates between cows receiving estradiol in the preovulatory period and the CON treatment in the time period from embryo transfer to maternal recognition of pregnancy, as estimated by GEPOOL. In the time period between maternal recognition of pregnancy and attachment, cows in the CON treatment lost 75% of existing pregnancies, while cows receiving estradiol lost 35% of existing pregnancies ($P = 0.04$). In the time following attachment before visualization of pregnancy by transrectal ultrasonography, none of the existing pregnancies in the estradiol treated cows were lost while one-half of existing pregnancies in the CON treated cows were lost. Exposure to estradiol in the preovulatory period, whether in the form of ECP or EB, increased the odds of pregnancy on d 29 ($P < 0.005$) and increased the likelihood of a positive pregnancy prediction by PSPB on d 24 ($P = 0.05$), as shown in Table 2. Each of the additional pregnancy prediction outcomes by treatment are listed in Table 2, however no other pregnancy predictions were affected by treatment.

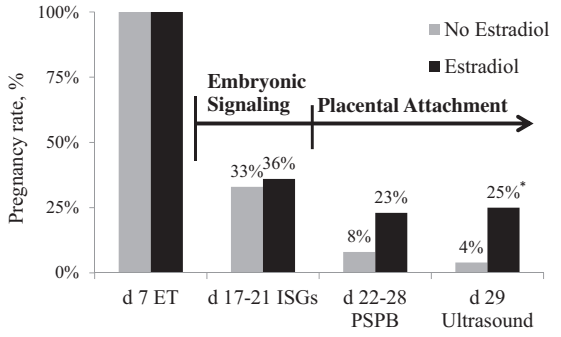

Fig. 4. Stages of pregnancy loss as evidenced by pregnancy rate on d 7, d 17–21 (ISG expression of *ISG1, MX2*, and *OAS1*), d 22–28 (PSPBPool), and visualization of fetal heartbeat by transrectal ultrasonography on d 29 (*$P < 0.05$).

4. Discussion

Exogenous estradiol injections provided different preovulatory estradiol exposure to the EB, ECP, and CON treated cows, in agreement with Souza et al. (2005). Cows receiving ECP displayed elevated circulating estradiol concentrations beginning 4–8 h after injection that were sustained until simulated ovulation. Cows receiving EB displayed a rapid and 2–3 fold greater increase in circulating estradiol concentrations than ECP cows which was also sustained until simulated ovulation. Cows receiving CON treatment generated no elevated circulating estradiol concentrations.

Reduced serum progesterone concentrations among estradiol treated cows in the current study were not expected but may have been related to changes in steroid

hormone metabolism associated with estradiol exposure. Progesterone concentrations were similar to those reported by Fields et al. (2009) and should have been sufficient to maintain pregnancies. Use of ovariectomized cows in the current study eliminated the deleterious effects of luteolysis on pregnancy, thus progesterone profiles achieved with this supplementation should have resulted in pregnancy retention to d 29.

Exogenous estradiol also influenced timing and peak concentrations of an LH surge. Cows treated with ECP had two surges of LH the first 25 h after treatment with ECP and the second after treatment with GnRH. This is a shorter interval than the 42 h interval from ECP treatment to the LH surge reported by Stevenson et al. (2004); however, a greater dose of ECP was utilized in the present study. Cows in both the EB and CON treatments had a LH surge following treatment with GnRH. Timing of LH surges in each treatment suggests that the estradiol agonists ECP and EB must be metabolized to estradiol before having biological activity. Precise interval to estrus following ECP and EB injections was not recorded in the current study.

Interferon-τ (IFNT) production by the trophoblastic cells of the elongating embryo beginning on d 13–16 is obligatory for maternal recognition of pregnancy (Roberts et al., 1992). Interferon-τ is also known to up regulate a class of genes known as interferon stimulated genes (ISG) in maternal leukocytes, which have practical applications for pregnancy detection (Yankey et al., 2001; Gifford et al., 2007; Green et al., 2010). The present study examined ISG expression of *ISG15*, *MX2*, and *OAS1* on d 17, 19, and 21 as markers of pregnancy success and as a tool to identify those cows that did not receive adequate trophoblastic signaling to maintain pregnancy. Expression of *ISG15*, *MX2*, and *OAS1* was increased on d 17, 19, and 21 in cows in the current study that were later diagnosed as pregnant by ultrasound on d 29, in agreement with others (Han et al., 2006; Gifford et al., 2007; Green et al., 2010). Increased expression of *ISG15* and *MX2* in pregnant cows on d 21 compared to d 17 is consistent with the results by Green et al. (2010) during a similar time frame, but unlike results of Green et al. (2010) *OAS1* expression did not change over time in the present study. Correlations between the interferon stimulated genes in the present study were similar to those of Green et al. (2010). Changes in expression of ISGs among the 59 observations when cows did not remain pregnant until d 29 in the current study were used with other measures in determining the timing of embryonic loss.

Robinson et al. (2006) compared *IFNT* expression in bovine trophoblasts and intrauterine concentrations of IFNT between pregnant and non-pregnant cows on d 14, 16, and 18 after ovulation. Among pregnant cows, uterine IFNT concentrations increased from d 14 to 18 and was positively correlated with embryonic size. Since neither ISG expression itself nor discriminate analysis pregnancy predictions using ISG expression differed between estradiol and CON treatment on d 17, 19 and 21 in the current study (Table 2), it suggests that preovulatory estradiol exposure either does not induce differences in growth rate of the embryo, or the effects of preovulatory estradiol exposure are not manifested during the trophoblastic signaling period.

Concentration of PSPB in maternal circulation has been shown to be as accurate as ultrasonography for pregnancy detection in cattle (Szenci et al., 1998). Identified in the placental membranes of pregnant cows (Butler et al., 1982), PSPB is produced by the trophoblastic binucleate cells as early as d 15 of pregnancy and released into maternal circulation during placental attachment. Sasser et al. (1986) quantified PSPB by RIA and demonstrated its potential usefulness as a pregnancy detection mechanism as early as d 24 however, the protein functions more reliably as a pregnancy marker on d 28–30 (Szenci et al., 1998). Furthermore, PSPB concentrations decreased in circulation on the same day as embryonic death (Semambo et al., 1992). Thus, PSPB concentration was chosen as a marker of pregnancy success and placental attachment in the current study. Pregnancy rates did not differ across treatments during d 17–21, based on ISG expression, but pregnancy rates were greater for estradiol treated cows on d 24 (based on PSPB concentrations). Thus, preovulatory estradiol is either necessary for continued embryonic growth after d 21 of gestation or is needed for embryo attachment.

Ultrasonography performed on d 29 revealed that cows that received estradiol (either EB or ECP) during a simulated estrus had greater pregnancy maintenance than cows that did not receive exogenous estradiol (CON). Since no treatment effect was found during the trophoblastic signaling period but began to favor estradiol-treated cows during the implantation period, and continued to impact pregnancy maintenance to d 29, it would appear that continued embryo growth necessary for placental attachment was compromised. The fact that ISG expression did not differ between treatments on d 17, 19 or 21 suggests that embryos in the CON group were viable to signal maternal recognition of pregnancy, but placental attachment had not become very intimate since detection of PSPB was decreased in the CON group. An interpretation of this data would be that embryos in cows not receiving estradiol during proestrus/estrus died between maternal recognition of pregnancy (d 17–21) and pregnancy confirmation by ultrasonography of d 29.

The use of ovariectomized cows in the present study allowed us to demonstrate the importance of estradiol during the preovulatory period on survival of embryos transferred on d 7, and pregnancy establishment. Cows that were exposed to estradiol in the preovulatory period were more likely to maintain pregnancy to d 29. Thus, at least a portion of the beneficial effect of increased proestrous interval and ovulatory follicle diameter on pregnancy success is due to the effects of a concurrent increase in estradiol on uterine environment. The exact mechanism by which this is achieved is not clear, but the timing of embryonic loss appears to be around d 22–24 of gestation. Uniform exogenous progesterone supplementation and similar quality embryos provided all cows equal opportunities to remain pregnant and allowed us to test the hypothesis that estradiol was necessary for embryo survival and pregnancy establishment. The uterine environment of cows that were not exposed to elevated estradiol prior to progesterone treatment was less capable of supporting a pregnancy to d 29. The critical period of pregnancy loss in estradiol deficient cows was around d 22–24 during placental

attachment. We suggest that estradiol exposure during the preovulatory period is necessary to sustain embryonic growth and/or placental attachment. Because ovariectomized cows were used in this model and pregnancy rates were lower than anticipated, it is also possible that ovarian factors produced during the luteal phase other than progesterone are involved in continued embryo growth and pregnancy establishment. It could be speculated that the rise in estradiol associated with follicular dominance around the time of maternal recognition of pregnancy may provide additional signaling in the uterus during pregnancy establishment.

Conflicts of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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