Theriogenology xxx (2015) 1–11

#869

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Expression of estrus modifies the gene expression profile in reproductive tissues on Day 19 of gestation in beef cows

S. Davoodi [a], R.F. Cooke [b], A.C.C. Fernandes [a], B.I. Cappellozza [b], J.L.M. Vasconcelos [c], R.L.A. Cerri [a,*]

[a] Faculty of Land and Food Systems, The University of British Columbia, Vancouver, British Columbia, Canada
[b] Eastern Oregon Agricultural Research Center, Oregon State University, Burns, Oregon, USA
[c] Faculdade de Medicina Veterinária e Zootecnia, UNESP, Botucatu, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 22 June 2015
Received in revised form 22 September 2015
Accepted 1 October 2015

Keywords:
Conceptus
CL
Endometrium
Estrus
Gene expression
Preimplantation

ABSTRACT

The aim of this study was to test the effect of expression of estrus at artificial insemination (AI) on endometrium, conceptus, and CL gene expression of beef cows. Thirty-six multiparous nonlactating Nelore cows were enrolled on an estradiol- and progesterone (P4)-based timed AI protocol (AI = Day 0) and then slaughtered for the endometrium, CL, and conceptus collection on Day 19. The animals were retrospectively grouped on the basis of cows that (1) showed signs of estrus near AI (n = 19; estrus) and (2) did not show any signs of estrus (n = 17; nonestrus). Body condition score, blood sampling, and ultrasound examination were performed on Days 0, 7, and 18 of the experiment followed by messenger RNA extraction and quantitative reverse transcription polymerase chain reaction analysis of 58 target genes. Data were checked for normality and analyzed by ANOVA for repeated measures using proc GLM, MIXED, and UNIVARIATE of SAS. Only pregnant cows were included in the analyses (n = 12; nonestrus, n = 11). Estrous expression had no correlation with parameters such as body condition score, preovulatory follicle and CL diameter, P4 concentration in plasma on Days 7 and 18 after AI, and interferon-tau concentration in the uterine flushing ($P > 0.15$); however, a significant increase was observed in conceptus size from cows that expressed estrus ($P = 0.02$; 38.3 ± 2.8 vs. 28.2 ± 2.9 mm). The majority of transcripts affected by estrous expression in the endometrium belong to the immune system and adhesion molecule family (*MX1*, *MX2*, *MYL12A*, *MMP19*, *CXCL10*, *IGLL1*, and *SLPI*; $P \leq 0.05$), as well as those related with prostaglandin synthesis (*OTR* and *COX-2*; $P \leq 0.05$). Genes related to apoptosis, P4 synthesis, and prostaglandin receptor were downregulated (*CYP11A*, *BAX*, and *FPr*; $P < 0.05$) in the CL tissue of cows that expressed estrus. In addition, four genes were identified as differentially expressed in the 19-day-old conceptus from cows that expressed estrus (*ISG15*, *PLAU*, *BMP15*, and *EEF1A1*; $P < 0.05$). There was also a significant effect of Day 7 concentration of P4 mainly affecting the immune system, adhesion molecules, and wnt signaling pathway of the endometrium (*IGLL1*, *MX2*, *SLPI*, *TRD*, *APC*, *WNT2*, *GLYCAM1*, and *MYL12A*; $P < 0.05$). A significant interaction between estrous expression and P4 concentration on Day 7 was more pronounced in immune system genes (*MX1*, *MX2*, *TRD*, *SLPI*, and *IGLL1*; $P < 0.05$). This study reported that estrous expression at the time of AI favorably altered the gene expression profile in reproductive tissues during the preimplantation phase toward a more receptive state to the elongating conceptus. These effects seem to be more evident in the endometrium during the time of dynamic remodeling for embryo implantation.

* Corresponding author. Tel.: +1 604 822 5056; fax: +1 604 822 6394.
E-mail address: ronaldo.cerri@ubc.ca (R.L.A. Cerri).

0093-691X/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2015.10.002

1. Introduction

Early and late embryonic loss occurs mainly in the first 6 weeks of gestation and is responsible for major losses in the beef and dairy industry. A great proportion of these embryonic losses occur between Days 8 and 21 after fertilization [1,2]. The effect of changes in steroid hormone concentrations is critical as they affect the ability of the endometrium to receive and maintain the conceptus. Previous studies have reported the correlation between the concentration of estradiol (E2) in plasma and the ovulation, increased pregnancy/artificial insemination (AI), and decreased pregnancy loss in beef and dairy cattle [3,4].

Estradiol initiates crucial modifications in the endometrium environment such as increased epithelial cell height and ciliation in the fimbria [5] and ampulla [6]. Indeed, E2 concentrations during the proestrus period are positively correlated with the diameter of the preovulatory follicle, subsequent CL diameter, concentration of progesterone (P4) during diestrus [7], and conception rates in dairy cows [8,9]. Pereira et al. [10] also reported that a shorter proestrus duration decreased conception rates even when embryo transfer technology was used. Furthermore, an increase in pregnancy maintenance from Days 7 to 27 after AI was observed when serum E2 concentration on Day 0 and P4 concentration on Day 7 were greater in recipient cows [11].

Comparing transcriptome of the receptive and nonreceptive endometrium has led to identifying signaling pathways involved in embryonic growth and development [12]. Before implantation, during the receptivity phase of the endometrium, specific genes related to the immune system, adhesion molecules, and developmental genes are extensively regulated [12,13]. Some of these genes are activated once the conceptus starts secreting interferon-tau (IFNT), but the timing of this activation varies considerably.

Immunologically, the embryo is an allograft for the dam and more specifically for the uterine tissue. Therefore, a complex modulation of immune cells and its signals are necessary to allow the maintenance of the conceptus. The uterus is an immunologically privileged site [14], and E2 has shown to play an important role by upregulating *SERPINA14* messenger RNA (mRNA) synthesis during estrus [15]. On the basis of studies performed in sheep [16], this serpin family member has immunomodulatory roles which include (1) blocking T cell proliferative responses [17], (2) impairing natural killer cell activity [18], and (3) decreasing antibody production [19]. A second group of genes critical for the survival of the early embryo are related to cell adhesion. Proper attachment and invasion of the embryo in the endometrium depend on adhesion-related molecules. In ruminants, the fetal tissue invades [20] the endometrium and establishes a synepitheliochorial type of placentation [21]. Apposition, adhesion, and invasion performed by the conceptus are controlled by the endometrium [22]. Studies have found upregulation of some adhesion molecules such as *SPP1* and *GLYCAM1* during the implantation phase in ruminants [23,24]. The canonical wnt signaling pathway, which is regulated by sexual steroids including E2 [25], is critical for morphogenesis and development of the preimplantated conceptus [26,27]. The wnt regulatory role in embryonic development is still unknown as previous studies have shown that the wnt activation improves [28], reduces [29], or has no effect [30] on the proportion of embryos that can develop to the blastocyst stage.

The function of the CL and consequent P4 synthesis in the preimplantation phase is key for proper embryo elongation and IFNT synthesis. However, it is unclear whether estrous expression could further modify the transcriptome of the CL. It is reasonable to believe that a fully mature preovulatory follicle could improve the chances for a more developed CL.

The objective of this study is to test the effects of behavioral expression of estrus before AI on gene expression of target transcripts in the endometrium, CL, and conceptus on Day 19 of gestation. We hypothesized that expression of estrus is associated with a complete maturation and function of the preovulatory mechanisms, therefore improving the transcriptome profile in reproductive tissues during the preimplantation phase.

2. Materials and methods

2.1. Animals and housing

Thirty-six nonlactating multiparous Nelore cows (body condition score [BCS] = 5.5 ± 0.1) [31] were assigned to an estrous synchronization plus timed AI protocol [32] (Fig. 1). All animals were cycling and with absence of any clinical disorder. The animals were between 48 and 72 months of age. The animals were not lactating at the time of study, and previous parturition occurred 300 to 360 days before enrollment. The cows were enrolled onto a synchronization protocol that was carried out as follows: 2-mg injection of estradiol benzoate (Estrogin; Farmavet, São Paulo, SP, Brazil) and a second-use (previously used for 9 days) intravaginal P4-releasing device (CIDR, originally

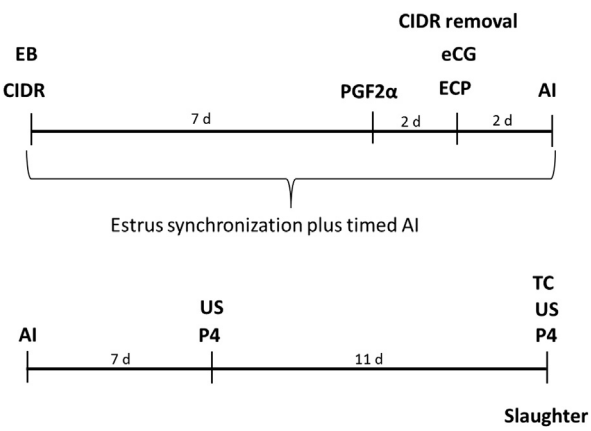

Fig. 1. Diagram of study. Cows received a 2 mg injection of estradiol benzoate (EB, Estrogin; Farmavet, São Paulo, SP, Brazil) and a second-use intravaginal progesterone-releasing device (CIDR, originally containing 1.9 g of progesterone; Zoetis, São Paulo, Brazil) on study Day −11, a 12.5-mg injection of PGF2α (Lutalyse; Zoetis, São Paulo, Brazil) on Day −4, CIDR removal in addition to 0.6 mg of estradiol cypionate (ECP; Zoetis, São Paulo, Brazil) and 300 IU of eCG (Novormon, Schering-Plough Co., São Paulo, Brazil) on Day −2, and timed artificial insemination (AI) on Day 0. P4, blood collection for progesterone analysis, TC, tissue collection; US, ultrasonographic examination of ovaries.

containing 1.9 g of P4; Zoetis, São Paulo, Brazil) on study Day −11, a 12.5-mg injection of PGF2α (Lutalyse; Zoetis) on Day −4, CIDR removal in addition to 0.6 mg of estradiol cypionate (Zoetis) and 300 IU of eCG (Novormon; Schering-Plough Co., São Paulo, Brazil) on Day −2, and timed AI on Day 0. All cows were inseminated on Day 0 by the same technician, using semen from the same bull and batch. The cows were maintained in a single *Brachiaria brizantha* pasture (10 ha) with ad libitum access to forage and water. All animals received a 100 g of a protein–mineral mix + 100 g of ground corn per cow daily (on an as-fed basis).

The cows were observed for behavioral expression of estrus by visual observation twice a day for 30 minutes each from the administration of the PGF2α injection until timed AI. The cows were visually observed for mounting activity and secondary signs of estrus (e.g., chin rest, following, vaginal mucus, swollen vulva) and then clustered in two different groups (1) estrus (n = 19), when cows expressed evident signs of estrus the day before (PM) and/or the day of AI (AM), and (2) nonestrus (n = 17), for cows that did not show any signs of estrus. To clearly define the subgroups, only animals that were positive (estrus) or negative (nonestrus) for mounting activity and secondary signs of estrus were considered for this study (n = 36), whereas animals that were positive for only primary or secondary signs were removed from the project. Only pregnant cows were then included in the analyses (estrus, n = 12; nonestrus, n = 11).

2.2. Blood samples and ultrasound examinations

Blood samples were collected immediately before AI (Day 0) and on Days 7 and 18 of the experiment via jugular venipuncture into commercial blood collection tubes (Vacutainer, 10 mL; Becton Dickinson, Franklin Lakes, NJ, USA) containing 158 USP units of freeze-dried sodium heparin. After collection, the blood samples were placed immediately on ice, centrifuged (2500 × *g* for 30 minutes, 4 °C) for plasma harvest, and stored at −20 °C on the same day of collection for further analysis of P4 using an ELISA procedure according to manufacturer's guidelines (Ovucheck Plasma Elisa Kit; Biovet Inc., Saint Hyacinthe, Québec, Canada). Transrectum ultrasonography (7.5-MHz transducer, 500 V; Aloka, Wallingford, CT, USA) was performed concurrently with blood sampling on Days 0, 7, and 18 to verify ovulation and CL development. Corpus luteum volume was calculated using the formula for volume of a sphere: volume = $4/3\pi \times (D/2)^3$, where D is the maximum luteal diameter. All animals analyzed had a preovulatory follicle with the absence of a CL on Day 0, confirmed ovulation on Day 7 (presence of a CL in the ipsilateral ovary of the preovulatory follicle observed on Day 0), and a CL greater than 0.38 cm^3 in volume on Days 7 and 18.

2.3. Slaughter and tissue collection

The cows were slaughtered on Day 19 after timed AI, and reproductive tracts were immediately collected, placed on ice, and processed for collection of the conceptus, uterine luminal flushing, and tissue samples from the CL and endometrium on the basis of the procedures described by Bilby et al. [33]. More specifically, the uterine horn ipsilateral to the CL was isolated from the reproductive tract, and the ovary containing the CL was removed. The CL was incised with a scalpel for collection of luteal tissue. Subsequently, 20 mL of saline were injected into the uterotuberal junction of the selected uterine horn, massaged gently, and exited through an incision at the tip of the uterine horn. Uterine luminal flushing media and the conceptus were recovered in a sterile 100 by 15-mm Petri dish. The conceptus was measured for length and weight, whereas the uterine luminal flushing was stored in a 15-mL sterile conical tube (Corning Life Sciences, Tewksbury, MA, USA) for further analysis of IFNT concentrations using a bovine-specific commercial ELISA kit (MyBioSource LLC, San Diego, CA, USA). The selected uterine horn was then cut along the mesometrial border, and samples of the endometrium were collected. After collection, the conceptus, as well as luteal and endometrial samples, were stored in 5-mL sterile cryogenic tubes (CRAL Artigos para Laboratórios, Cotia, São Paulo, Brazil) containing 2 mL of RNA stabilization solution (RNAlater; Ambion Inc., Austin, TX, USA), maintained at 4 °C for 24 hours, and stored at −20 °C until further processing.

2.4. RNA extraction

Total RNA was extracted from samples using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA). The tissue:Trizol ratio (mg:mL) was 100:1 for all samples (1-mL TRIzol per 50- to100-mg tissue). Quantity and quality of isolated RNA were assessed UV absorbance (NanoDrop 2000; UV-Vis Spectrophotometer; Thermo scientific, Wilmington, DE, USA) at 260 nm and 260:280-nm ratio, respectively. Extracted total RNA was stored at −80 °C until further processing.

2.5. Primer design

All forward and reverse primers were designed from bovine mRNA sequences (National Center for Biotechnology Information) using the PrimerQuest PCR Design Tool (Integrated DNA Technologies, Coralville, IA, USA). The primer sequence, product length, and gene accession number are provided in Table 1.

2.6. Reverse transcription synthesis of cDNA

After extraction, reverse transcription reactions were performed by following the kit manufacturer's protocol. A total RNA sample of 2500 ng was treated with 1-μL DNase (New England Biolabs, Ipswich, MA, USA) to digest any DNA left from the RNA extraction and were incubated for 10 minutes at 75 °C. Next, to prevent DNAse I activity by chelating the divalent cations that it requires (Mg^{++} and Ca^{++}), and also to prevent cation-related RNA cleavage, 0.25-μL EDTA, ultrapure 0.5 M, PH 8.0 (Life Technologies, Burlington, ON, USA) was added to each sample and incubated for 10 minutes at 37 °C. When DNase treatment finished, a High Capacity cDNA Reverse Transcription Kit

Table 1
Primer sequences of analyzed genes from endometrium, CL, and conceptus tissues.

Gene symbol	Accession no.	Primer	Primer sequence	Product length (bp)
Sequences of primers used for qPCR analysis of endometrium tissue				
GAPDH	NM_001034034.2	F	GAG ATC CTG CCA ACA TCA A	83
		R	CTT CTC CAT GGT AGT GAA GAC	
LGALSBP3	NM_001046316.2	F	CTC TGT CTC CTG GTC TTT	127
		R	GGG ATT GGA CTT GGA GTA	
SERPINA14	NM_174821.2	F	GAC AGA GTC ACC TCA GAT A	91
		R	CAT CGA GAA TAC CTC CTT TC	
CLD4	NM_001014391.2	F	CCC TCA TCG TCA TCT GTA T	99
		R	CCT TGG AGC TCT CAT CAT	
IDO	NM_001101866.2	F	AGC TAT GGT CTC CTT GAG	121
		R	GCC TCC AGT TCC TCT ATT	
MSX1	NM_174798.2	F	AAG CAG TAC CTG TCC ATC	88
		R	GGT TCT GAA ACC AGA TCT TC	
SPP1	NM_174178.2	F	GGA CTT CAC ATC ACA CAT AG	97
		R	CTC GCT ACT GTT GGT TTC	
IL-10	NM_174088.1	F	GCT CAG CAC TAC TCT GTT	97
		R	GTT GGC AAG TGG ATA CAG	
AXIN1	NM_001191398.1	F	GCC ATC TAC CGC AAA TAC	93
		R	CGA GAT GCA GTC CTT TAT G	
IGLL1	NM_001083800.1	F	GGA AGC AGC ACG AAT ATC	99
		R	GGG TCG ATA CTT ATC TTC ATA G	
TIMP2	NM_174472.4	F	GGT CAC GGA GAA GAA CAT	126
		R	TCC TCG ATG TCC AGA AAC	
MX2	NM_173941.2	F	CCA ATC AGA TCC CGT TCA	115
		R	TGA AGC AGC CAG GAA TAG	
TRD	XM_603355.3	F	GTC GCT TGT TTG GTG AAG	104
		R	CCA GGT GAG ATG GCA ATA	
CDH1	NM_001002763.1	F	CTG AGA ACG AGG CTA ATG T	132
		R	GGT CTG TGA CGA CGA TAA A	
RELN	NM_001206458.1	F	GGG TGT GCC AAT CAA TTC	100
		R	CTG GGT AAC AGC CTT CTT	
EMMPRIN	NM_001075371.2	F	GGT CAC CAT CAT CTT CAT CTA	73
		R	AGA GCC TAT GTC TTC ATC ATC	
LIFR	NM_001192263.1	F	GCT CTT GGA ATG GGA AAT AG	98
		R	CCA GAC TGA GAT GAG TTA CA	
SLPI	NM_001098865.2	F	GCC TTG GAG ATG AGA AAC	96
		R	GGT CCA GAC ATT CAG TTC	
MYL12A	NM_001015640.2	F	CAC CAT TCA GGA GGA TTA C	100
		R	GTC AAT AGG TGC TTC TCT G	
MYH10	NM_174834.1	F	GAC TAC CAG CGT GAA TTA G	115
		R	CCT GCA ACT GAA GGA TTT	
MYH9	NM_001192762.1	F	GAC AAG AGT GGC TTT GAG	96
		R	GTT CAC CTT CAC CTT CTT C	
IGHG1	DQ452014.1	F	GAC CCT CTG TCT TCA TCT	146
		R	GTT TAC CTC CAC GTT GTC	
FDZ4	NM_001206269.1	F	GTT CCA TCT GGT GGG TTA TTC	106
		R	GCT GCG ATG TGG AAA TAA GA	
FZD8	XM_005214320.1	F	CCT ATA TGC CCA ACC AGT TC	122
		R	CAT GCT GCA CAG GAA GAA	
WNT3	NM_001206024.1	F	AGA AGC GGA AGG AGA AGT	83
		R	CAC GTC ATA GAT GCG GAT AC	
AXIN2	NM_001192299.1	F	GGA GAA ATG CGT GGA TAC TT	103
		R	GTA GAT CGC TTT GGC TAC TC	
GSK3B	NM_001101310.1	F	GGG TCA TTT GGT GTC GTG TAT C	97
		R	GAT CTG GAG CTC TCG GTT CTT A	
GLYCAM1	NM_174828.2	F	CCT CTG CTC AGT TCA TCA GG	97
		R	TCT GAT CAC AAT TTG CTC TTT GG	
SELL	NM_001076141.1	F	GGT GGG AAC CAA CAA ATC	86
		R	CAC AGT CCT CCT TAC TCT TC	
WNT2	NM_001013001.1	F	TCC TGT GAC CCA AAG AAG	98
		R	GCA AAC TTG ATC CCA TAG TC	
CXCL10	NM_001046551.2	F	GTG TAC CTC TCT CTA GGA ATA C	107
		R	GGA TTG ACT TGC AGG AAT G	
PTX3	NM_001076259.2	F	CGC TGA TGC TGT GAT TTC	101
		R	CCA CCG AGT CAC CAT TTA	
DKK1	NM_001205544.1	F	CCA TGG GCT GGA GAT ATT	100
		R	GTG AAG CCT GGA AGA ATT AC	
MMP19	NM_001075983.1	F	ATC TTG AAC CTA CCG TCT AC	83
		R	GCC ACA TTG CTC CAA TAC	

(*continued on next page*)

Table 1 (*continued*)

Gene symbol	Accession no.	Primer	Primer sequence	Product length (bp)
APC	NM_001075986.2	F	GAG CCC TTC ACA GAA TGA	118
		R	CTC AGG ATA CAC GGG ATA AG	
FZD7	NM_001144091.1	F	GGG TGT GCC AAT CAA TTC	138
		R	CTG GGT AAC AGC CTT CTT	
CTNNB1	NM_001076141.1	F	CCC TTT GTC CAG CAA ATC	119
		R	CTG TGT TCC ACC CAT AGA	
MX1	NM_1733940.2	F	AGT CCA TCC GAC TAC ATT TC	102
		R	CTT CTT CTG CCT CCT TCT C	
COX-2	NM_174445	F	AGGTGTATGTATGAGTGTAGGA	484
		R	GTGCTGGGCAAAGAATGCAA	
Sequences of primers used for qPCR analysis of CL tissue				
BAX	NM_173894.1	F	TCT GAC GGC AAC TTC AAC TG	98
		R	CCA TGA TGG TCC TGA TCA ACT C	
CYP11A1	NM_176644.2	F	GAA TTA CCC AGG CAT CCT CTA C	97
		R	TCT CCG TAA TAT TGG CCT TGA C	
BCL-2	NM_001166486.1	F	ATC GTG GCC TTC TTT GAG TTC	104
		R	TCA GGT ACT CGG TCA TCC AC	
NOS2	NM_001076799.1	F	GAG CTT CTA CCT CAA GCT ATC G	94
		R	TCT ATC TCC TTT GTT ACT GCT TCC	
NOS3	NM_181037.3	F	GAT GGT CAA CTA CAT CCT GTC C	100
		R	GGT CTT CTT CCT GGT GAT GC	
FGF2	NM_174056.3	F	CAA CAG AAG ACC TAG GGA AGA C	124
		R	ACA GCC AAC TCC TAA CAT CC	
StAR	NM_174189.2	F	TAC ACC ATG TGG AAT GTC AGG	104
		R	CCT GTG TCA GTT GTA CAG TCT C	
3BHSD	NM_174343.3	F	GGT AAC GTG GCC TGG ATG	123
		R	CTT GTA GGG CGA GTT GTC ATA G	
FPr	D17395	F	TTAGAAGTCAGCAGCACAG	98
		R	ACTATCTGGGTGAGGGCTGATT	
OXT	M25648.1	F	GTCTGCACCATGGCAGGTT	125
		R	CAGGGGGCAGTTCTGAATGT	
Sequences of primers used for qPCR analysis of embryo tissue				
PLAU	NM_174147.2	F	CTA GGG AGA AAG AAG AGT TCC	125
		R	TCG ATG CCT CCT GTA GAT	
HOXB7	NM_174342.2	F	ACC TAC ACC CGC TAT CA	118
		R	TGA TCT GTC TTT CTG TGA GG	
FTH1	NM_174062.3	F	AGG TGG AAG CCA TCA AAG	102
		R	GGG TGT GCT TGT CAA AGA	
EEF1A1	NM_174535.1	F	CTG GAA GAT GGC CCT AAA T	102
		R	GGG AGG ATA ATC AGA GAA GC	
GPX4	NM_174770.3	F	GCT GGC TAT AAC GTC AAA TTC	91
		R	GCT GGA CTT TCA TCC ATT TC	
ISG15	NM_174366.1	F	GTA CAA GCA GAC CAG TTC	84
IL-6	NM_173923.2	F	CTT CAA ACG AGT GGG TAA AG	97
		R	TAC TTC ATC CGA ATA GCT CTC	
BMP15	NM_001031752.1	F	CAT ACA GAC CCT GGA CTT TC	108
		R	GAG AGG TGG GAA TGA GTT AG	
IFN-tau	AF238612	F	GCCCTGGTGCTGGTCAGCTA	102
		R	CTT CAT GAG GCC GTA TTC	

Abbreviations: F, forward; qPCR, quantitative polymerase chain reaction; R, reverse.

(Applied Biosystems, Foster City, CA, USA) was used to synthesize complementary DNA (cDNA) from RNA. To proceed for reverse transcription polymerase chain reaction (RT-PCR) master mix, 5 μL of DNase-treated RNA was mixed with a 5-μL reaction mixture containing 1 μL of 10X random primers, 0.4 μL of 0.8-mM deoxyribonucleoside triphosphate mixture, 1 μL of 10X buffer, 0.5 μL of 50 U/μL of reverse transcriptase, 0.25 μL of 40,000 U/mL of RNase inhibitor (New England Biolabs), and 1.85 μL of nuclease-free water (provided in the kit). Then, the mixture was centrifuged at 2000 rpm for 2 minutes at 4 °C. The conditions used for RT-PCR was set as follows: 37 °C for 30 minutes, 75 °C for 15 minutes, and 4 °C for the final step. Finally, the products were stored at −20 °C until the quantification polymerase chain reaction (qPCR) was performed.

2.7. Quantitative real-time PCR

To perform transcription analysis and gene expression of reproductive tissues, 58 genes in total were selected on the basis of evidence in the literature showing their impact on endometrium remodeling, CL function, and embryo survival: 39 genes for endometrium, 10 genes for CL, and 9 genes for the conceptus (Table 1). These genes have been grouped on the basis of their roles during endometrium preparation, embryo and CL development (Table 2).

Transcript abundance was compared for a set of genes in the endometrium, CL, and embryonic tissue with three replicates per sample using quantitative real-time PCR (qPCR). The qPCR analysis was performed using the Rotor-Gene Q real-time cycler (Qiagen, Hilden, Germany),

Table 2
Flowchart of gene function.

Endometrium			CL			Conceptus	
Cell adhesion	Immune system	Growth and development	Apoptosis	Angiogenesis	Steroid biosynthesis	Morphogenesis	Maternal recognition
MMP19	*IGLL1*	*CTNNB1*	*BCL2*	*NOS2*	*StAR*	*PLAU*	*IFNT*
CLD4	*SELL*	*WNT2*	*BAX*	*NOS3*	*FPr*	*HOXB7*	*ISG15*
GLYCAM1	*CXCL10*	*DKK1*		*FGF2*	*3βHSD*	*BMP15*	
TIMP2	*PTX3*	*AXIN1*			*OXT*	*GPX4*	
SPP1	*TRD*	*AXIN2*			*CYP11A*	*EEF1A1*	
LGALSBP3	*MX2*	*APC*				*IL6*	
SERPINN	*MX1*	*FZD7*				*FTH1*	
EMMPRIN	*IL10*	*GSK3β*					
CDH1	*IDO*	*MSX1*					
MYH9	*LIFR*	*RELN*					
MYH10	*IGHG1*	*FZD8*					
MYL12A	*SLPI*	*WNT3*					
		FZD4					
PGF2α biosynthesis							
COX2 *OTR*							

Genes analyzed in this study have been grouped according to their function in the endometrium, CL, and conceptus development.

SYBR Green Master Mix (QuantiFast SYBR Green PCR Kit; Qiagen, Toronto, Ontario, Canada), and gene-specific primers (Table 1). For each sample, the qPCR reaction consisted of 1.2 μL of cDNA, 0.2 μM of each forward and reverse primer, 7.5 μL of 2X SYBR green master mix, and 5.7 μL of RNase-free water in a final volume of 15 μL per reaction. Reaction conditions included an initial step of 95 °C (10 minutes), followed by 45 cycles of 95 °C (15 seconds) and 60 °C (45 seconds). Oligonucleotide sequences used to amplify segments of each gene tested are listed in Table 1. Only cycle thresholds smaller than 35 were used for analysis.

2.8. Statistical analysis

The quantitative RT-PCR results were analyzed using the mean threshold cycle (Ct) for each transcript. The Ct was calculated and normalized for the housekeeping gene *GAPDH* to generate delta (Δ) Ct values. Changes in relative abundance of specific transcripts were calculated by using the delta delta (ΔΔ) Ct method [34]. Differences between cows that expressed estrus and those that did not express estrus at the time of AI were analyzed using the PROC MIXED procedure of SAS software (version 9.2; SAS Institute Inc., Cary, NC, USA). Univariable analyses were performed using BCS, CL size, P4 concentration, embryo length, follicle size, and IFNT concentration, but only the significant ones were included in the final model (treatment was forced into the final model).

3. Results

3.1. Endometrium gene expression

The results from endometrium gene transcription analysis showed that genes related to the immune system, *IGLL1, CXCL10, MX1, MX2*, and *SLPI* had significant fold differences when comparing estrus with nonestrus cows. *MX1, MX2*, and *SLPI* showed 1.6, 2.1, and 2.0 fold increases, respectively ($P = 0.02$, 0.03, and 0.01), in estrus cows. In addition, within this category, *IGLL1* and *CXCL10* had tendency for a 1.8- and 1.4-fold increase, respectively ($P = 0.10$), in estrus cows. From the adhesion category, *MMP19* and *MYL12A* were upregulated with 1.5 ($P = 0.05$) and 1.3 ($P = 0.1$) fold increases, respectively, in estrus cows. *OXT* and *COX-2,* which belong to the prostaglandin biosynthesis group, showed a 0.6- and 0.4-fold downregulation in gene expression, respectively, when comparing the estrus with the nonestrus group. The fold change differences of genes in the endometrium are shown in Figure 2.

Transcript levels of other immune system–related genes (*PTX3*, *TRD*, *IL10*, *IDO*, *IGHG1*, and LIFR) quantified from the endometrium were not statistically significant ($P > 0.15$). Cell adhesion–related genes, *CLDD4*, *GLYCAM1*, *TIMP2*, *SPP1*, *LGALSBP3*, *SERPINA14*, *EMMPRIN*, *MYH9*, and *MYH10,* showed no significant difference between estrus and nonestrus cows in their mRNA levels ($P > 0.15$). The mRNA expression levels of developmental genes mostly linked with the wnt signaling pathway (*AXIN1*, *APC*, *FZD7*, *CTNNB1*, *WNT2*, *DKK1*, *GSK3B*, *FZD8*, *FZD4*, *WNT3*, *AXIN2*, *RELN*, and *MSX1*) were not different between estrus and nonestrus cows ($P > 0.15$).

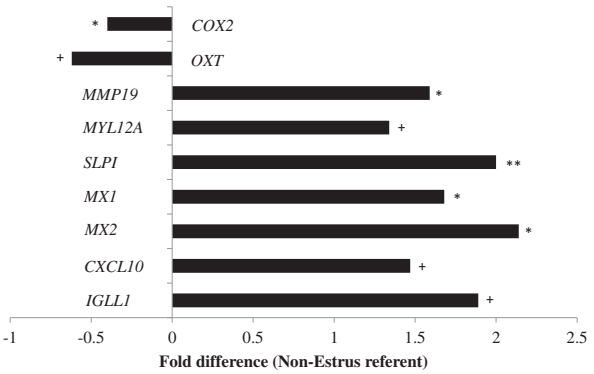

Fig. 2. Effect of estrous expression on endometrium gene expression. Significant fold difference based on nonestrus expression as a referent has been shown for genes with significant pattern of expression in endometrium tissue. For this graph, the asterisks (*, **) and (+) refer to $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.10$, respectively.

3.2. Corpus luteum gene expression

Among the analyzed genes from the CL tissue, *FPr* ($P = 0.05$), *CYP11A* ($P = 0.01$), and BAX ($P = 0.05$) were significant downregulated in estrus cows, with a 0.7-, 0.7-, and 0.8-fold difference in mRNA expression, respectively. The remaining genes analyzed (*NOS2*, *NOS3*, *FGF2*, *OXT*, *3BHSD*, *StAR*, and *BCL2*) were statistically unaltered by estrous expression ($P > 0.20$). All values of fold increase and significance are depicted in Figure 3.

3.3. Gene expression in the embryo associated with estrus

Downregulation in two different groups was observed in the embryos collected from cows in the estrus group compared with the nonestrus group. The *ISG15* gene, from the maternal recognition of the pregnancy group, was observed a 0.56-fold decrease ($P = 0.05$) in embryos collected from estrus cows. The *eFF1A1* ($P = 0.09$) and *PLAU* ($P = 0.01$), both transcripts that belong to the morphogenesis group, were also different between groups with a 0.81- and 0.74-fold difference, respectively, when comparing embryos from estrus versus nonestrus cows. A fold difference of 0.19 was observed for *BMP15*, which was downregulated in estrus cows compared with nonestrus cows ($P = 0.10$; Fig. 4). The remaining genes (*HOXB7*, *FTH1*, *IL6*, *IFNT*) were not significantly different (Fig. 4).

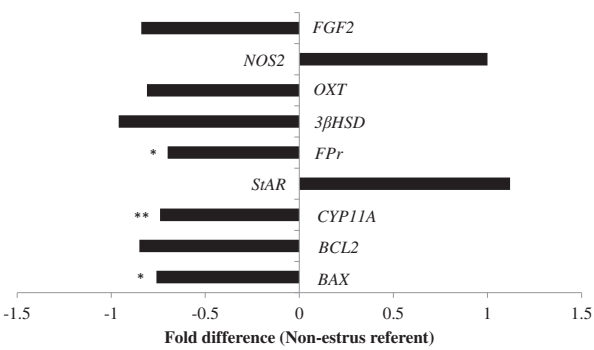

Fig. 3. Effect of estrous expression on CL genes involved in steroidogenesis, angiogenesis, and apoptosis. Significant fold difference based on nonestrus expression as a referent has been shown for genes with significant pattern of expression in CL tissue. For this graph, the asterisks (*) and (**) refer to $P \leq 0.05$ and $P \leq 0.01$, respectively.

3.4. Ovarian and embryo parameters

Estrous expression positively affected the dimensional development of the embryos ($P = 0.02$) as they were around 10 cm longer when collected from cows in the estrus group (Table 3). The IFNT concentration within the uterine flushing media was not different between the estrus and nonestrus groups ($P = 0.47$). Follicle size was not affected by estrous expression as well (Table 3; $P = 0.89$). The CL tended to be smaller ($P = 0.10$) although concentrations of P4 were not statistically significant when comparing estrus and nonestrus cows on Day 7 ($P = 0.34$; Table 3). By Day 18, the volume of the CL was not different between groups ($P = 0.45$). There was a tendency for a greater BCS in nonestrus cows compared with estrus cows ($P = 0.10$; Table 3).

3.5. Effect of concentration of P4 on Day 7

Effect of concentration of P4 (high and low; based on median value) on Day 7 as a main factor affecting gene expression was analyzed. Gene expression in the endometrium was affected by P4 concentration. Immune-related genes within the endometrium such as *TRD*, *IGLL1*, *MX2*, and *SLPI* showed a significant upregulation when comparing the high versus low P4 concentrations ($P < 0.05$; Fig. 5). Other groups of genes which showed upregulation in the high-P4 group compared with the low-P4 group belong to adhesion molecules (*GLYCAM1* [$P = 0.003$] and *MYL12A* [$P = 0.02$]), the wnt signaling pathway (*APC* [$P = 0.001$] and *WNT2* [$P = 0.01$]). The *IL10* ($P = 0.09$), *CXCL10* ($P = 0.07$), *MX1* ($P = 0.07$), and *CDH1* ($P = 0.09$) also showed a tendency for upregulation in the high-P4 group compared with the low-P4 group. Embryo gene expression was also not affected by concentration of P4 on Day 7. The interaction between estrus effect and P4

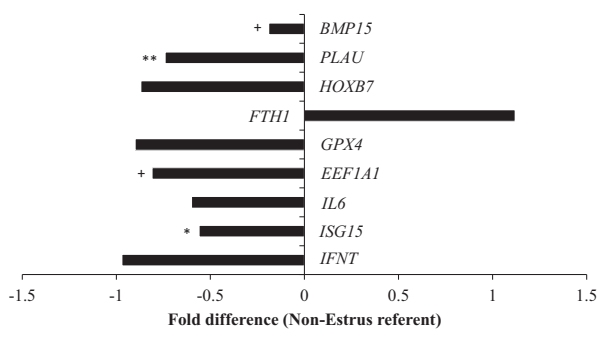

Fig. 4. Effect of estrous expression on embryo genes involved in morphogenesis, immune system, and protein synthesis. Significant fold difference based on nonestrus expression as a referent has been shown for genes with significant pattern of expression in endometrium tissue. For this graph, the asterisk (*, **) and (+) refer to $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.10$, respectively.

Table 3
Reproductive parameters collected on Days 7 and 19 of pregnancy from cows in the estrus and nonestrus groups.

Parameters	Estrus cows	Nonestrus cows	P value
BCS (1–5 scale)	3.30 ± 0.10	3.45 ± 0.10	0.10
Follicle diameter (mm)	14.0 ± 1.0	14.2 ± 1.0	0.89
P4 on Day 7 (ng/mL)	3.8 ± 0.9	5.2 ± 1.0	0.34
P4 on Day 18 (ng/mL)	3.9 ± 0.7	4.4 ± 0.8	0.62
CL diameter on Day 7 (cm)	6.9 ± 0.8	8.8 ± 0.8	0.10
CL diameter on Day 18 (cm)	10.5 ± 1.0	9.4 ± 1.0	0.45
Embryo length (cm)	38.3 ± 2.8	28.2 ± 2.9	0.02
IFNT concentration (pg/mL)	8.3 ± 1.7	10.2 ± 1.9	0.47

Abbreviations: BCS, body condition score; IFNT, interferon-tau; P4, progesterone.

concentration on Day 7 and their synergistic effect on endometrium gene expression were significant for immune-related genes such as *MX1* (P = 0.003), *MX2* (P = 0.04), *TRD* (P = 0.05), and *SLPI* (P = 0.003). *GLYCAM1* (P = 0.04), *APC* (P = 0.01), and *IGLL1* (P = 0.08; Fig. 6) also showed a differential gene expression on the basis of the interaction between expression of estrus and concentration of P4 on Day 7.

Other nongenomic results showed that IFNT concentration, CL volume on Days 7 and 18, follicle diameter, and BCS were not affected by categorization based on concentration of P4 on Day 7 (P > 0.15).

3.6. Effect of conceptus size

Animal variables and embryo gene expression were analyzed against embryo size (large and small; based on medium length [34 cm]). Embryo size did not affect IFNT concentration, CL volume on Days 7 and 18, concentration of P4 on Days 7 and 18, follicle diameter, and BCS. There were only two conceptus transcripts downregulated in the large-conceptus group (*BMP15* [fold difference = 0.05; P = 0.005] and *GPX4* [fold difference = 0.81; P = 0.05]).

4. Discussion

The aim of this study was to investigate the association of estrous expression at the time of AI with expression of

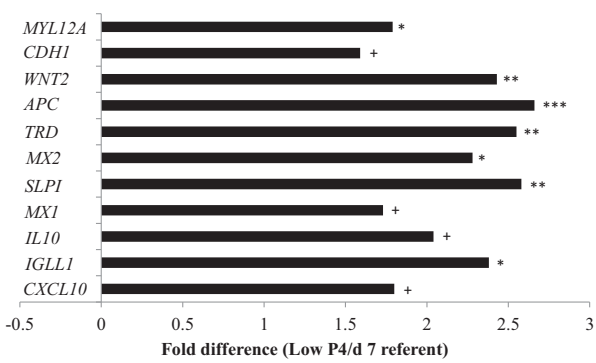

Fig. 5. Effect of progesterone (P4) concentration at Day 7 on endometrium gene expression. Significant fold difference based on the low-P4 group as a referent has been shown for genes with significant pattern of expression in endometrium tissue. For this graph, the asterisks (*, **, ***) and (+) refer to P ≤ 0.05, P ≤ 0.01, P ≤ 0.001, and P ≤ 0.10, respectively.

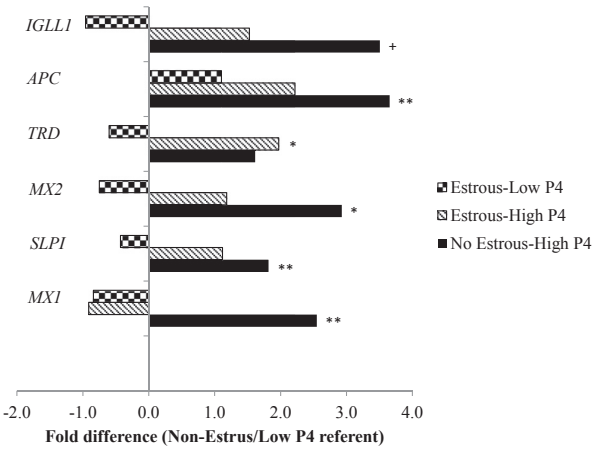

Fig. 6. Interaction between estrous expression and concentration of progesterone (P4) on Day 7 on endometrium gene expression. For this graph, the asterisks (*, **) and (+) refer to P ≤ 0.05, P ≤ 0.01, and P ≤ 0.10, respectively.

critical genes in the endometrium, CL, and embryo during the preimplantation period. In addition, the difference in estrous expression was evaluated for reproductive parameters such as CL volume, conceptus size, concentration of P4 in plasma, and follicle diameter. Evidence from this study supports our hypothesis that estrous expression positively influences the expression of target genes important for embryo survivability. Cows that expressed estrous behavior near AI had a significant improvement in the profile of endometrium gene expression critical for suppressing the local maternal immune system and adhesion between endometrium epithelial cells and the conceptus, as well as partly inhibiting the mRNA machinery for PGF2α synthesis. Genes related to the immune system and adhesion group in the endometrium were also significantly affected by P4 concentration on Day 7. The results from the gene analysis of the CL also confirmed downregulation of cellular pathways associated with apoptosis and PGF2α synthesis which favors CL maintenance and secretion of P4, both key to sustain pregnancy.

The early embryonic development until implantation is arguably the most important period that define a successful pregnancy. A significant proportion of all embryonic losses in lactating cows occurs between Days 8 and 21 of pregnancy [35]. Because of operational limitations, it was not possible to check for length of dominance or P4 levels during the growth of the preovulatory follicle. The specific causes that lead to the presence or absence of estrous expression are unknown on the basis of the data collected in this study and warrant further investigations. The expression of estrus can indicate the state of sensitivity of the hypothalamus to E2 and perhaps the best timing for the optimal function of all other reproductive tissues related with the survivability of the early embryo.

The upregulation of immune system–related genes involved in endometrium receptivity (*MX1*, *MX2*, *IGLL1*, *SLPI*, and *CXCL10*) is in agreement with previous studies [36–39]. The *CXCL10* acts to attract trophoblasts to the endometrium and promote adhesive activity in ruminant

species [40,41] and has been shown to have more than a 11-fold upregulation in pregnant cows [39]. In a study by Walker et al. [42], *CXCL10* was downregulated in subfertile dairy cows compared with fertile cows. Myxoviruses are integral components of the innate immune system and were identified in blood leukocytes as a potential marker for pregnancy diagnosis in dairy heifers [43]. Hicks et al. [44] indicated a 15-fold increase in *MX1* and *MX2* from Days 12 to 15 after AI caused by pregnancy. Others have shown a temporal difference in the expression of these genes as indicated by greater expression of *MX2* on Days 18 and 20 compared with Days 14 and 16 of pregnancy [45]. The *IGLL1* expression positively impacts B cells development which are critical members of the adaptive immunity [46] and can indirectly enhance *MX1* and *MX2* activity. *SLPI* has the ability to interrupt the activation of transcription factor NF-kB and possibly cause a reduction in *COX-2* expression, favoring CL maintenance. Some studies showed that hypoxia-induced *COX-2* expression also happens through the NF-kB pathway [47,48].

The extensive molecular and structural changes taking place during the preimplantation stage in the endometrium are necessary for the reorganization of the glandular endometrium [49]. *MMP19* has been shown to be important for the regulation of conceptus attachment in bovine endometrium [50], whereas *MYL12A* expression is important for the regulation of protrusion and adhesion-generated signaling [51,52] as well as for cadherin clustering [53,54] and the stability of the cell–cell junction.

Data from the present study showed a decrease in the expression of *OTR* in the endometrium in the estrus group. It was reported that the expression of *OTR* is impacted by P4 and E2 concentrations [55,56] and key for the synthesis of PGF2α and consequent maintenance of the CL [50,55,57,58]. The downregulation of *COX2*, a major enzyme necessary for the synthesis of PGF2α, is probably a product of the lower expression of *OTR*. The optimal reduction in the expression of *OTR* and *COX2* signals on Day 19 of the estrous cycle may only appear when the complete estrous cycle, including proper expression of estrus, is allowed.

Results regarding the role of the wnt signaling pathway showed no significant difference in gene expression between animals that did or did not express estrus at the time of AI. The influence of the wnt signaling pathway could be dependent on the stage of embryo development. The activation of wnt signaling in bovine embryos by inhibitors of GSK3β either blocks or increases development to the blastocyst stage [29]. It is known that at the morula stage, the embryo undergoes major genome activation [59] and perhaps the wnt signaling may have been already deactivated on Day 19 of pregnancy.

Analysis of target genes in the CL showed a significant decrease in genes related to apoptosis, PGF2α and P4 synthesis. Downregulation of *BAX* may be due to the anti-luteolytic effects of *IFNT* (increase) or *COX2* (decrease). Sugino et al. [60] reported high *BCL2* and low *BAX* expression in the CL during the midluteal phase and early pregnancy in humans, whereas low *BCL2* and high *BAX* expression were found in the regressing CL. The PGF2α receptors (*FPr*) are required to interact with PGF2α released from uterus at the time of luteal regression [61], but during pregnancy, the number of PGF2α receptors in CL is reduced to allow CL maintenance. The PGF2α synthesis is indirectly regulated by endometrial *COX2*, and its expression is necessary before luteolysis [62–64], which is corroborated by the results of the present study.

The gene expression of the conceptus had a significant reduction in *ISG15* and *PLAU* expression in the estrus group. In addition, *eEF1A1* and *BMP15* showed a tendency for downregulation. *ISG15* synthesis is stimulated by IFNT secretion from the conceptus and early detected on Day 17 of pregnancy but with peak levels between Days 18 and 23 and back to baseline levels by Day 45 in cows [65]. No difference between estrus and nonestrus cows regarding IFNT concentration on Day 18 conceptus tissue was observed in the present study, in spite of the difference in conceptus length favoring the estrus group. The benefit of a larger conceptus is likely the physical occupation of the lumen and increased likelihood of promoting IFNT-driven changes in as much endometrium tissue as possible. Although in some studies, they have reported a correlation between IFNT secretion and embryo size [66], they have not observed a relationship between IFNT concentration or embryo size and *IFNT* mRNA expression. We also observed a reduction in *BMP15* expression of cows in the estrus group which possibly relates to the tempospatial genome activation of the embryo. In a study by Pennetier et al. [67], these authors found *BMP15* transcripts until the five- to eight-cell stage but only trace levels in the morulae stage. According to our results, cows with smaller embryo size had greater expression of *BMP15* and *GPX4* in estrus versus nonestrus cows. The target genes affected by estrous expression in the conceptus seem of significant importance, but their interpretation is rather unclear. Further studies are necessary to clarify their roles and relationship with the endometrium status.

Ultimately, the present study found a correlation between P4 concentration and endometrial gene expression, which was mainly pronounced in immune system–related genes (*IL-10*, *MX1*, *SLPI*, *MX2*, *TRD*, *CXCL10*, and *IGLL1*), adhesion molecules (*GLYCAM1*, *CDH1*, and *MYL12A*), and wnt signaling (*APC* and *WNT2*). Other variables such as conceptus gene expression or animal physiological factors were not affected by P4 concentration on Day 7 of gestation. There was an interaction between estrous expression and P4 concentration which significantly affected expression of genes in the endometrium, specifically when the combination of estrous expression and low P4 concentration was in place. The upregulation of critical groups of genes in the endometrium under these circumstances of estrous expression and low P4 could be of great importance, particularly in beef cows. It is likely that a combination of factors leading to the day of collection (e.g., expression of estrus, endocrine milieu during the preimplantation phase) leads to the optimal function of reproductive tissues and embryonic receptivity.

4.1. Conclusions

The expression of estrus promoted changes in the preimplantation endometrium, CL, and conceptus gene expression. Critical cellular pathways related to

suppression of the maternal immune system, attachment between the conceptus and the endometrium, and CL maintenance during pregnancy were favorably expressed in cows that expressed estrus near AI. Moreover, cows in the estrus group yielded longer conceptuses, which can be associated with better chances of survival. The effects of expression of estrus seem to interact with P4 concentration on Day 7 of the estrous cycle in a way that positively influences endometrium receptivity and embryo development.

Acknowledgments

This study was supported by a contribution from the Dairy Research Cluster Initiative (Grant no. AIP-CL04; Dairy Farmers of Canada, Agriculture and Agri-Food Canada, Canadian Dairy Network, and Canadian Dairy Commission) and from the Natural Sciences and Engineering Research Council of Canada (Grant no. 418672-13). B.I. Cappellozza was funded by the Science Without Borders program from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Brasília, Brazil). A.C.C. Fernandes was funded by the PDSE-CAPES program from the Brazilian Ministry of Education. The authors also thank all students who assisted with the data collection.

References

[1] Dunne LD, Diskin MG, Sreenan JM. Embryo and foetal loss in beef heifers between day 14 of gestation and full term. Anim Reprod Sci 2000;58:39–44.
[2] Roche JF, Bolandl MP, McGeady TA. Reproductive wastage following artificial insemination of heifers. Vet Rec 1981;109:401–4.
[3] Lopes AS, Butler ST, Gilbert RO, Butler WR. Relationship of preovulatory follicle size, estradiol concentrations and season to pregnancy outcome in dairy cows. Anim Reprod Sci 2007;99:34–43.
[4] Pereira MHC, Rodrigues ADP, Martins T, Oliveira WVC, Silveira PSA, Wiltbank MC, et al. Timed artificial insemination programs during the summer in lactating dairy cows: comparison of the 5-d Cosynch protocol with an estrogen/progesterone-based protocol. J Dairy Sci 2013;96:6904–14.
[5] Murray MK. Changes in secretory status, cell height and percentage ciliation of epithelial lining of sheep fimbria oviduct during early pregnancy. J Reprod Fertil 1996;106:173–83.
[6] Murray MK. Epithelial lining of the sheep ampulla oviduct undergoes pregnancy-associated morphological changes in secretory status and cell height. Biol Reprod 1995;53:653–63.
[7] Bridges GA, Mussard ML, Pate JL, Ott TL, Hansen TR, Day ML. Impact of preovulatory estradiol concentrations on conceptus development and uterine gene expression. Anim Reprod Sci 2012;133:16–26.
[8] Geary TW, Smith MF, MacNeil MD, Day ML, Bridges GA, Perry GA, et al. Triennial reproduction symposium: influence of follicular characteristics at ovulation on early embryonic survival. J Anim Sci 2013;91:3014–21.
[9] Vasconcelos JL, Sartori R, Oliveira HN, Guenther JG, Wiltbank MC. Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology 2001;56:307–14.
[10] Pereira MH, Sanches CP, Guida TG, Rodrigues AD, Aragon FL, Veras MB, et al. Timing of prostaglandin F2α treatment in an estrogen-based protocol for timed artificial insemination or timed embryo transfer in lactating dairy cows. J Dairy Sci 2013;6:2837–46.
[11] Atkins JA, Smith MF, MacNeil MD, Jinks EM, Abreu FM, Alexander LJ, et al. Pregnancy establishment and maintenance in cattle. J Anim Sci 2013;91:722–33.
[12] Spencer TE, Sandra O, Wolf E. Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. Reproduction 2008;135:165–79.
[13] Spencer TE, Bazer FW. Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. Biol Reprod 1995;53:1527–43.
[14] Bainbridge DR. Evolution of mammalian pregnancy in the presence of the maternal immune system. Rev Reprod 2000;5:67–74.
[15] Ulbrich SE, Frohlich T, Schulke K, Englberger E, Waldschmitt N, Arnold GJ, et al. Evidence for estrogen-dependent uterine serpin (SERPINA14) expression during estrus in the bovine endometrial glandular epithelium and lumen. Biol Reprod 2009;81:795–805.
[16] Moffatt J, Bazer FW, Hansen PJ, Chun PW, Roberts RM. Purification, secretion and immunocytochemical localization of the uterine milk proteins, major progesterone-induced proteins in uterine secretions of the sheep. Biol Reprod 1987;36:419–30.
[17] Tekin S, Padua MB, Brad AM, Rhodes ML, Hansen PJ. Expression and properties of recombinant ovine uterine serpin. Exp Biol Med (Maywood) 2006;231:1313–22.
[18] Tekin S, Hansen PJ. Natural killer-like cells in the sheep: functional characterization and regulation by pregnancy-associated proteins. Exp Biol Med (Maywood) 2002;227:803–11.
[19] Skopets B, Liu WJ, Hansen PJ. Effects of endometrial serpin-like proteins on immune responses in sheep. Am J Reprod Immunol 1995;33:86–93.
[20] Pfarrer C, Hirsch P, Guillomot M, Leiser R. Interaction of integrin receptors with extracellular matrix is involved in trophoblast giant cell migration in bovine placentomes. Placenta 2003;24:588–97.
[21] Wooding FB. The role of the binucleate cell in ruminant placental structure. J Reprod Fertil Suppl 1982;31:31–9.
[22] Von Rango U. Fetal tolerance in human pregnancy—a crucial balance between acceptance and limitation of trophoblast invasion. Immunol Lett 2008;115:21–32.
[23] Bazer FW, Kim J, Song G, Satterfield MC, Johnson GA, Burgardt RC, et al. Uterine environment and conceptus development in ruminants 2012;2:297–304.
[24] Forde N, Mehta JP, McGettigan PA, Mamo S, Bazer FW, Spencer TE, et al. Alterations in expression of endometrial genes coding for proteins secreted into the uterine lumen during conceptus elongation in cattle. BMC Genomics 2013;14:321.
[25] Van der Horst PH, Wang Y, van der Zee M, Burger CW, Blok LJ. Interaction between sex hormones and WNT/β-catenin signal transduction in endometrial physiology and disease. Mol Cell Endocrinol 2012;358:176–84.
[26] Atli MO, Guzeloglu A, Dinc DA. Expression of wingless type (WNT) genes and their antagonists at mRNA levels in equine endometrium during the estrous cycle and early pregnancy. Anim Reprod Sci 2011;125:94–102.
[27] Macdonald LJ, Sales KJ, Grant V, Brown P, Jabbour HN, Catalano RD. Prokineticin 1 induces Dickkopf 1 expression and regulates cell proliferation and decidualization in the human endometrium. Mol Hum Reprod 2011;17:626–36.
[28] Aparicio IM, Garcia-Herreros M, Fair T, Lonergan P. Identification and regulation of glycogen synthase kinase-3 during bovine embryo development. Reproduction 2010;140:83–92.
[29] Lim KT, Gupta MK, Lee SH, Jung YH, Han DW, Lee HT. Possible involvement of Wnt/β-catenin signaling pathway in hatching and trophectoderm differentiation of pig blastocysts. Theriogenology 2013;79:284–290.e1–2.
[30] Xie H, Tranguch S, Jia X, Zhang H, Das SK, Dey SK, et al. Inactivation of nuclear Wnt-beta-catenin signaling limits blastocyst competency for implantation. Development 2008;135:717–27.
[31] Wagner JJ, Lusby KS, Oltjen JW, Rakestraw J, Wettemann RP, Walters LE. Carcass composition in mature Hereford cows: estimation and effect on daily metabolizable energy requirement during winter. J Anim Sci 1988;66:603–12.
[32] Meneghetti M, Filho OGS, Peres RFG, Lamb GC, Vasconcelos JLM. Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows I: basis for development of protocols. Theriogenology 2009;72:179–89.
[33] Bilby TR, Guzeloglu A, Kamimura S, Pancarci SM, Michel F, Head HH, et al. Pregnancy and bovine somatotropin in nonlactating dairy cows: I. Ovarian, conceptus, and insulin-like growth factor system responses. J Dairy Sci 2004;87:3256–67.
[34] Cooke FNT, Pennington KA, Yang Q, Ealy AD. Several fibroblast growth factors are expressed during pre-attachment bovine conceptus development and regulate interferon-tau expression from trophectoderm. Reproduction 2009;137:259–69.
[35] Diskin MG, Morris DG. Embryonic and early foetal losses in cattle and other ruminants. Reprod Domest Anim 2008;43(Suppl 2):260–7.

[36] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 2001;25:402–8.
[37] Forde N, Duffy GB, McGettigan PA, Browne JA, Mehta JP, Kelly AK, et al. Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. Physiol Genomics 2012;44:799–810.
[38] Bauersachs S, Wolf E. Immune aspects of embryo-maternal cross-talk in the bovine uterus. J Reprod Immunol 2013;97:20–6.
[39] Cerri RLA, Thompson IM, Kim IH, Ealy AD, Hansen PJ, Staples CR, et al. Effects of lactation and pregnancy on gene expression of endometrium of Holstein cows at day 17 of the estrous cycle or pregnancy. J Dairy Sci 2012;95:5657–75.
[40] Imakawa K, Imai M, Sakai A, Suzuki M, Nagaoka K, Sakai S, et al. Regulation of conceptus adhesion by endometrial CXC chemokines during the implantation period in sheep. Mol Reprod Dev 2006;73:850–8.
[41] Nagaoka K, Nojima H, Watanabe F, Chang K, Christenson R, Sakai S, et al. Regulation of blastocyst migration, apposition, and initial adhesion by a chemokine, interferon gamma-inducible protein 10 kDa (IP-10), during early gestation. J Biol Chem 2003;278:29048–56.
[42] Walker CG, Littlejohn MD, Mitchell MD, Roche JR, Meier S. Endometrial gene expression during early pregnancy differs between fertile and subfertile dairy cow strains. Physiol Genomics 2012;44:47–58.
[43] Stevenson JL, Dalton JC, Ott TL, Racicot KE, Chebel RC. Correlation between reproductive status and steady-state messenger ribonucleic acid levels of the myxovirus resistance gene, MX2, in peripheral blood leukocytes of dairy heifers. J Anim Sci 2007;85:2163–72.
[44] Hicks BA, Etter SJ, Carnahan KG, Joyce MM, Assiri AA, Carling SJ, Kodali K, Johnson GA, Hansen TR, Mirando MA, Woods GL, Vanderwall DK, Ott TL. Expression of the uterine Mx protein in cyclic and pregnant cows, gilts, and mares. J Anim Sci 2003;81:1552–61.
[45] Green JC, Okamura CS, Poock SE, Lucy MC. Measurement of interferon-tau (IFN-tau) stimulated gene expression in blood leukocytes for pregnancy diagnosis within 18-20d after insemination in dairy cattle. Anim Reprod Sci 2010;121:24–33.
[46] Bauer TR, McDermid HE, Budarf ML, Van Keuren ML, Blomberg BB. Physical location of the human immunoglobulin lambda-like genes, 14.1, 16.1, and 16.2. Immunogenetics 1993;38:387–99.
[47] Lukiw WJ, Ottlecz A, Lambrou G, Grueninger M, Finley J, Thompson HW, et al. Coordinate activation of HIF-1 and NF-kappaB DNA binding and COX-2 and VEGF expression in retinal cells by hypoxia. Invest Ophthalmol Vis Sci 2003;44:4163–70.
[48] Schmedtje JF, Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. J Biol Chem 1997;272:601–8.
[49] Wathes DC, Wooding FB. An electron microscopic study of implantation in the cow. Am J Anat 1980;159:285–306.
[50] Bauersachs S, Mitko K, Ulbrich SE, Blum H, Wolf E. Transcriptome studies of bovine endometrium reveal molecular profiles characteristic for specific stages of estrous cycle and early pregnancy. Exp Clin Endocrinol Diabetes 2008;116:371–84.
[51] Cai Y, Biais N, Giannone G, Tanase M, Jiang G, Hofman JM, et al. Nonmuscle myosin IIA-dependent force inhibits cell spreading and drives F-actin flow. Biophysical J 2006;91:3907–20.
[52] Vicente-Manzanares M, Zareno J, Whitmore L, Choi CK, Horwitz AF. Regulation of protrusion, adhesion dynamics, and polarity by myosins IIA and IIB in migrating cells. J Cell Biol 2007;176:573–80.
[53] Shewan AM, Maddugoda M, Kraemer A, Stehbens SJ, Verma S, Kovacs EM, et al. Myosin 2 is a key Rho kinase target necessary for the local concentration of E-cadherin at cell-cell contacts. Mol Biol Cell 2005;16:4531–42.
[54] Ivanov AI, Bachar M, Babbin BA, Adelstein RS, Nusrat A, Parkos CA. A unique role for nonmuscle myosin heavy chain IIA in regulation of epithelial apical junctions. PLoS One 2007;2:e658.
[55] Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M. Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev 2007;19:65.
[56] Bazer FW, Burghardt RC, Johnson GA, Spencer TE, Wu G. Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways. The content of the paper was presented at the V Jubilee Congress of the Society for Reproductive Biology in Wrocław, Poland. Reprod Biol 2008;8:179–211.
[57] Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC, Bayless K. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. Mol Hum Reprod 2010;16:135–52.
[58] Dorniak P, Bazer FW, Spencer TE. Prostaglandins regulate conceptus elongation and mediate effects of interferon tau on the ovine uterine endometrium. Biol Reprod 2011;84:1119–27.
[59] Memili E, First NL. Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. Zygote 1999;8:87–96.
[60] Sugino N, Suzuki T, Kashida S, Karube A, Takiguchi S, Kato H. Expression of Bcl-2 and Bax in the human corpus luteum during the menstrual cycle and in early pregnancy: regulation by human chorionic gonadotropin. J Clin Endocrinol Metab 2000;85:4379–86.
[61] Tomac J, Cekinović Đ, Arapović J. Biology of the corpus luteum. Periodicum Biologorum 2011;113:43–9.
[62] Parent J, Villeneuve C, Fortier MA. Evaluation of the contribution of cyclooxygenase 1 and cyclooxygenase 2 to the production of PGE 2 and PGF 2 in epithelial cells from bovine endometrium. Reproduction 2003;126:539–47.
[63] Charpigny G, Reinaud P, Tamby JP, Créminon C, Martal J, Maclouf J, et al. Expression of cyclooxygenase-1 and -2 in ovine endometrium during the estrous cycle and early pregnancy. Endocrinology 1997;138:2163–71.
[64] Arosh JA. Expression of cyclooxygenases 1 and 2 and prostaglandin e synthase in bovine endometrial tissue during the estrous cycle. Biol Reprod 2002;67:161–9.
[65] Austin KJ, Carr AL, Pru JK, Hearne CE, George EL, Belden EL, et al. Localization of ISG15 and conjugated proteins in bovine endometrium using immunohistochemistry and electron microscopy. Endocrinology 2004;145:967–75.
[66] Robinson RS, Fray MD, Wathes DC, Lamming GE, Mann GE. In vivo expression of interferon tau mRNA by the embryonic trophoblast and uterine concentrations of interferon tau protein during early pregnancy in the cow. Mol Reprod Dev 2006;73:470–4.
[67] Pennetier S, Uzbekova S, Perreau C, Papillier P, Mermillod P, Dalbiès-Tran R. Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15,andVASA in adult bovine tissues, oocytes, and preimplantation embryos. Biol Reprod 2004;71:1359–66.