Endometrial Inflammatory Cytokine Expression in Postpartum Beef Heifers Following Platelet Rich Plasma Treatment

By

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Abstract

Background: Uterine inflammation (e.g., endometritis) causes negative economic effects in the beef industry by reducing reproductive performance. Current treatments for endometritis in beef cattle involve hormones and antibiotics, which can be expensive and are often ineffective. The endometrial immune response regulates the release of cytokines and chemokines in response to inflammation. The objective of this research was measure endometrial pro-inflammatory cytokines (IL-6, TNFα) and chemokine (IL-8) expression in postpartum beef heifers following treatment with intrauterine platelet rich plasma (PRP), platelet poor plasma (PPP), or saline.
Methods: Twelve Angus-crossbred heifers calving under supervision were used for this study. Nine heifers calved without assistance (eutocia) and were divided equally into groups: PRP, PPP, and saline-treated eutocia (EUT; this group served as the control for all other groups). Three heifers needed assistance during calving (dystocia) and these heifers were put into a separate saline-treated group (DYS). Endometrial cells were collected at 2- and 4-weeks post-calving using a double-guarded endometrial swab. Intrauterine treatment was administered once after samples were collected 2-weeks post-calving. Total RNA was isolated from endometrial cells using a standard Trizol-chloroform protocol. The concentration and integrity of the RNA (RIN) was determined. Complementary DNA (cDNA) was prepared from the total RNA using a commercial kit. Forward and reverse primer sequences for IL-6, IL-8, and TNFα as well as the housekeeping gene (β-actin) were used as they had been previously validated for use in an endometrial gene expression study in dairy cattle. These primers were used in the current study for real-time polymerase chain reactions (PCR) to determine relative gene expression. Expression of each gene of interest (IL-6, IL-8, TNFα, DYS) relative to the expression of saline-treated controls (EUT) was calculated using the delta delta CT method. Differences between time

points (2- and 4-weeks post-calving) were determined using a one-way analysis of variance where significance was defined as $p<0.05$.

Results: RNA samples were partially degraded based upon RIN analysis, but cDNA creation resulted in good yields and PCR amplifications from all samples. Relative gene expression of IL-8 and IL-6 did not differ between time points in any treatment group. Relative gene expression of TNFα was reduced ($p=0.002$) at the second time point in the DYS group but not in the other groups.

Conclusion: Difficult calving increases the risk for developing endometritis but in healthy cattle, the increase in cytokine expression related to dystocia will resolve spontaneously. This explains why the relative expression of TNFα was higher 2-weeks post-calving compared to 4-weeks post-calving. This study also shows that intrauterine administration of platelet-rich plasma to normal calving heifers does not alter endometrial cytokine gene expression. Future studies will focus on investigating the effect of PRP treatment in cattle that experienced dystocia or have clinical evidence of endometritis.

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List of Acronyms

Acronym	Definition
DYS	Heifers that required assistance delivery their calves and that were administered intrauterine saline
EUT	Heifers that did not require assistance delivery their calves and that were administered intrauterine saline; this group served as the control for determining relative gene expression
IL	Interleukin
PCR	Polymerase chain reaction was used to synthesize cDNA from RNA
PPP	Platelet poor plasma
PRP	Platelet rich plasma
RIN	RNA integrity number
RTPCR	Real-time polymerase chain reaction was used to determine the relative gene expression within a sample
TNFα	Tumor necrosis factor-alpha

Introduction

Uterine Immune Response

Endometritis of cattle is a disease of the uterus categorized by an increased number of inflammatory cytokines correlated with epithelial attrition or necrosis and diffuse oedema of endometrium[1]. In most cases uterine diseases do not develop into greater reproductive problems, as a healthy and normal uterus is able to clear any bacterial infection efficiently. However, twenty percent of heifers' infections are not controlled and typically lead to chronic uterine inflammation[2]. The economic losses related to this disease are substantial, due to drop in yields, calves, and treatment costs[1,3]. In spite of current treatment involving hormones and antibiotics, no effective treatment helps decrease reproductive problems in beef heifers[4]. Recent studies have demonstrated that most of these medications are ineffective and lead to greater complications needing more antibiotics and more hormones increasing the cost of reproductive problems [5]. The inconsistency in the conclusions of these studies fail to demonstrate a reason for treating endometritis with antibiotics and hormones since it only adds extra cost. As consumers move toward antibiotic-free or hormone-free food, beef producers are looking for alternative treatments. Both antibiotics and hormones have withdrawal times to ensure none of this treatment resides in the meat prior to slaughter. Finding a treatment free of residues could be a helpful alternative for the beef industry.

The bovine uterus is made of the endometrium, myometrium and perimetrium[1] The endometrium is an intricate tissue that contains the tunica mucosa, lamina epithelialis and lamina propria, containing loose connective tissue and the tunica sub-mucosa of the uterus. In the lamina propria, neutrophils and lymphocytes are commonly detectable[1]. The inflammatory response is a

complicated activity involving many signaling cascades happening in this tract; the initial response of the endometrium against infection is reliant on the innate immunity and mucosal defense system. The uterine immune response is stipulated at the cellular level by uterine leukocytes and white blood cells that act as phagocytizing and clearing bacteria[1].

During calving, the uterus becomes exposed to foreign materials including feces and other microorganisms. Because of this exposure, bacterial contamination within the uterus is common immediately after calving[2]. The detection of bacterial invaders in the uterus first starts by the innate immune cell via toll-like receptors, which produce a tumor necrosis factor (**TNFα**) and a cascade of other pro-inflammatory cytokines known as interleukins (**IL**)[6]. Cytokines are signaling peptides; their primarily function is to protect the host from external threats such as bacteria and viruses when entering the body[7]. Tumor necrosis factor (**TNFα**) stimulates the chemokine interleukin-8 (**IL-8**). This chemoattractant stimulates polymorphonuclear cells (e.g., basophils, neutrophils) and monocytes to move directly to the site of infection[8]. Both TNFα and interleukin-6 (**IL-6**) are proinflammatory cytokines that arrive at the site of infection and promote inflammation, which then promote tissue repair by a signaling IL-8 to recruit more white blood cells[9]. Higher endometrial expression of TNFα following calving occurs in response to bacterial contamination in the uterus (especially after a difficult delivery)[10]. Endometritis results in the overexpression of TNFα, IL-6 and IL-8[6,11].

White blood cells are recruited by IL-8 to the endometrium during uterine inflammation. IL-6 is released primarily by monocytes and macrophages during cell injury, infection, and inflammation[10]. Both TNF and IL-6 cause can cause chronic inflammation[12]. With clinical endometritis, there is a significant influx of polymorphonuclear cells into the uterine lumen sufficient to result in the presence of purulent or mucopurulent exudate[1,13].

Current treatments for endometritis in beef cattle include antibiotics (ceftiofur, cephapirin, ampicillin, penicillin, and oxytetracycline) and hormones that stimulate uterine contractions (ecbolics). However, there was no significant difference in the resolution of chronic endometritis following treatments with uterine irrigation, estradiol and $PGF_{2\alpha}$ compared to no treatment[14,15,16]. Additionally both antibiotics and hormones have meat withdrawal times that need to be considered prior to slaughter[17].

Platelet Rich Plasma

Platelets are non-nucleated cell fragments derived from megakaryocytes located in the bone marrow[18]. Platelets are cytoplasmic fragments within the blood that are involved in coagulation, and which also possess limited bactericidal and phagocytic activity. Platelets contain several growth factors including platelet-derived growth factors, transforming growth factors, insulin-like growth factor, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor and hepatocyte growth factor[1,17]. Platelet rich plasma (**PRP**) is highly concentrated blood cell-free plasma containing 3 to 5 times the number of platelets than what is found in circulation[1]. Platelet granules contain numerous proteins, several growth factors, and a number of cytokines, chemokines, and antimicrobial peptides that participate in inflammatory processes that produce and release immunomodulatory factors[18]. The growth-promoting and anti-inflammatory factors in PRP speed up tissue regeneration and suppress the inflammatory process[1,19]. Intrauterine PRP has been used in dairy cattle to treat endometritis but it has not yet been tested in beef cattle[20]. Using PRP may be a cost effective, residue-free method for producers to treat subclinical endometritis in beef cattle.

Objective and Hypothesis

We hypothesized intrauterine PRP treatment will decrease IL-6, TNFα, and IL-8 expression in postpartum heifers. The objective of this research was to compare relative IL-6, IL-8, and TNFα gene expression after intrauterine treatment with platelet rich plasma (PRP), platelet poor plasma (**PPP**), and saline.

Materials and Methods

Animals and Treatment Groups

Twelve Angus-crossbred heifers calving under supervision were used for this study. Heifers calved at the Oregon State University (OSU) Hogg Animal Metabolism Building in February 2018. Nine heifers calved without assistance (eutocia). Three heifers had difficulty calving (dystocia) and needed assistance. Heifers and their calves were transported to the OSU Soap Creek Beef Ranch within 48 hours of calving. Two weeks after calving, heifers that calved without assistance were randomly divided into three equal groups: saline-treated (**EUT**), platelet poor plasma-treated (**PPP**), and platelet rich plasma-treated (**PRP**). Heifers that needed assistance during calving (**DYS**) also received saline treatment.

Endometrial cells were collected transcervically using a double-guarded endometrial swab at 2-weeks post-calving. Following endometrial cell collection, heifers received an intrauterine infusion of 10 mL of 0.9% sterile saline without preservative (EUT, DYS), sterile PRP, or sterile PPP depending upon their treatment group. Two weeks later (4 weeks post-calving), endometrial cells were collected in the same way as previously described.

RNA Isolation, Quantification, and Integrity Determination

After endometrial cell collection, endometrial swabs were cut with scissors to a length of 2 cm and put into RNAse-free microcentrifuge tubes containing 1 mL of Trizol reagent (Thermo Fisher Scientific, Eugene OR). Tubes were flash frozen in liquid nitrogen and transported from the ranch back to the university. Tubes were stored at -80°C until total RNA was isolated. Briefly, tubes were thawed on ice and vortexed briefly. Swabs were then removed with forceps

and discarded into a biohazard labeled container for Trizol® waste products. Chloroform (0.1 mL) was added each tube and the tubes were capped and briefly vortexed again. Then the microcentrifuge tubes were placed on ice for 2 minutes and centrifuged at 12,000 X g for 15 minutes at 4°C. The aqueous phase was then transferred to a fresh tube and RNA was precipitated by mixing with 0.25 mL isopropyl per 0.5 mL of Trizol (Thermo Fisher Scientific, Eugene, OR). Samples were incubated at 70°C for 10 minutes and centrifugation was repeated before removing the supernatant. The RNA was first washed with 1 mL 100% ethanol and then 70% ethanol. The final RNA pellet was dried for 15 minutes at room temperature and then dissolved in 20 µL of RNase-free water by repetitive gentle pipetting. The total RNA concentration and integrity was determined using the Agilent 2100 Bioanalyzer.

Generation of Complementary DNA

Complementary DNA was created using the Superscript IV VILO Master Mix with EZ-DNase kit (Thermo Fisher Scientific, Eugene OR). Following the manufacturer's protocol, RNA samples and the components of the cDNA kit were thawed ice, microcentrifuge tubes were labeled for the reverse transcription (**RT**) reaction and the "**no RT**" control reaction. Samples were briefly vortexed and then centrifuged. In a separate microcentrifuge tube, 10X EZ-DNase buffer and EZ-DNase enzyme were combined to create the digestion mix. The digestion mix was briefly vortexed and centrifuged. The digestion mix (2 µL) and RNAse-free water (8 µL) were added to each sample and mixed by pipetting. Samples were incubated at 37°C for 2 minutes. In another microcentrifuge tube, 150 µL of RNase-free water was added to 96 µL of the Superscript IV VILO Master Mix and mixed by pipetting. In another microcentrifuge tube, 150 µL of RNase-free water was added with 100 µL of Superscript IV VILO No RT and mixed by

pipetting. Both the RT and no RT master mixes were placed on ice. No RT reaction mix (10 µL) was added to each no RT sample tube. RT reaction mix (10 µL) was added to each RT tube. All samples were incubated at 25°C for 10 minutes, at 50°C for 10 minutes, and at 85°C for 5 minutes. All RT and no RT samples were stored at -20°C until used for real-time polymerase chain reaction (**RTPCR**).

Primers

Forward and reverse primer sequences for IL-6, IL-8, and TNFα as well as the housekeeping gene (β-actin) were commercially prepared (Thermo Fisher Scientific; **Table 1**). These primers had been previously validated for use in an endometrial gene expression study in dairy cattle[3]. Each primer was briefly centrifuged to bring the powder to the bottom of the tube and then reconstituted with 250 µL of TE buffer, vortexed briefly, and then re-centrifuged. A new microcentrifuge tube for each primer was labeled and each primer was diluted to concentration of 100 µM. In each new microcentrifuge tube 10 µL from stock solution was added to microcentrifuge tubes and diluted with 90 µL of TE buffer for a total 100 µL primer solution. Primers were stored at -20°C until used.

Real-Time Polymerase Chain Reaction

For each **RTPCR** experiment, cDNA samples, primers and RTPCR kit components were placed in ice to thaw, vortexed and then centrifuged briefly. For each RTPCR experiment, a 96-well plate layout was set up online using the Applied Biosystems 7500 software for the Fast Time PCR v206 (Applied Biosystems, Thermo Fisher Scientific). For each gene of interest, 2,450 µL of SYBR green and 1,862 µL of nuclease-free were added to a 14 mL sterile tube.

Forty-nine microliters of the forward and reverse primer for each gene of interest were added to the tube and then the tube containing the RTPCR master-mix was vortexed briefly. For each RTPCR reaction, 45 µL of master-mix was added to each well of a 96-well RTPCR 0.1 mL Fast PCR plate (Thermo Fisher Scientific). Then, 5 µL of each cDNA sample was added to each respective well and mixed with the master-mix in each well by pipetting without introducing bubbles. After adding the cDNA, the RTPCR plate was placed it into RTPCR thermocycler (Applied Biosystems Thermo Fisher Scientific) following the manufacturer's recommended cycling conditions (**Table 2)**. Each RTPCR reaction was run in duplicate.

Data Analysis

Expression of each gene of interest (IL-6, IL-8, TNFα, DYS) relative to the expression of saline-treated controls (EUT) was calculated using the delta delta CT method. Briefly, the average CT from each of duplicate RTPCR reactions was calculated. Then, the β-actin CT from each sample was subtracted from the gene of interest CT for each sample. This step was repeated for all three genes of interest (IL-8, IL-6, TNFα). Next, the average CT for the control group (EUT) was subtracted from the remaining CT value. In many cases, this was a negative value. The relative gene expression was then calculated by multiplying $2^{-(\text{remaining CT value})}$. Differences between time points (2- and 4-weeks post-calving) for each treatment group were determined using a one-way analysis of variance where significance was defined as $p<0.05$.

Table 1. Primer sequences for genes of interest and the housekeeping gene.

Gene	Primer Direction	Primer Sequence (5'-3')	Number of base pairs
IL-6	Forward	CCAGGAACGAAAGAGAGC	150
	Reverse	CAGAAGTCATCACCAGGAG	150
IL-8	Forward	CAAGAGCCAGAAGAAACCTGAC	223
	Reverse	AGTGTGGCCCACTCTCAATAAC	223
TNFα	Forward	CTCTTCTGCCTGCTGCACTTC	202
	Reverse	CCATGAGGGCATTGGCATACG	202
β-actin	Forward	AGGCATCCTGACCCTCAAGTA	95
	Reverse	GCTCGTTGTAGAAGGTGTGGT	95

Table 2. Real-time polymerase chain reaction cycling conditions used for this research.

Step	Temperature	Duration	Cycles
RT AmpliTac Gold Polymerase activation	95℃	10 minutes	Hold
Denature	95℃	15 seconds	40
Anneal	60℃	1 minute	

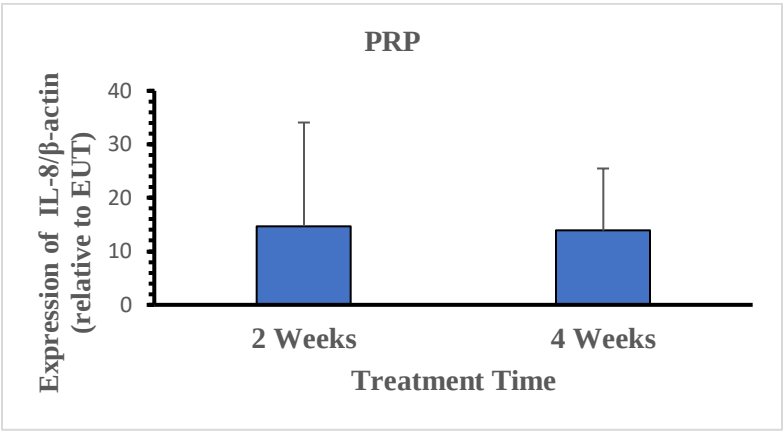
Results

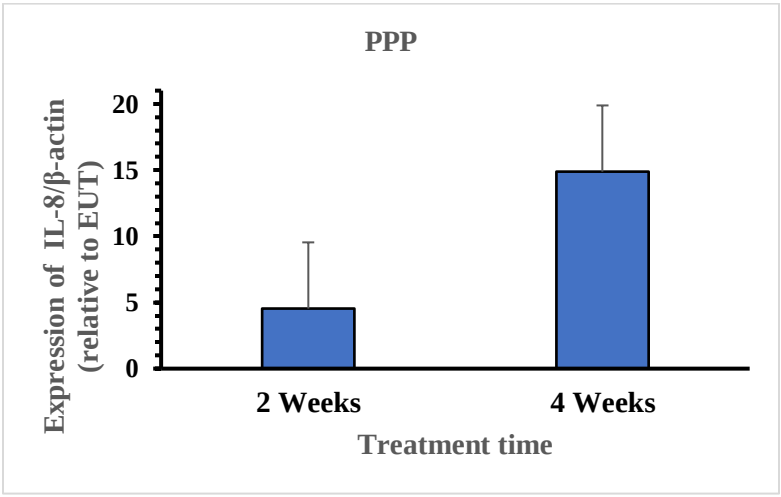
The RNA samples used in this experiment were partially degraded based upon an average RIN of 4.6 (range: 2.7- 6.1; **Table 3**), which is less than desirable for RTPCR experiments[20]. However, the cDNA creation from this RNA resulted in good yields and PCR amplifications from all samples.

Relative gene expression of IL-8 (**Figure 1**) and IL-6 (**Figure 2**) did not differ between time points in any treatment group (PRP: p=0.966 and p=0.108, respectively; PPP: p=0.449 and p=0.636, respectively; DYS: p=0.428 and p=0.120, respectively). Relative gene expression of TNFα (**Figure 3**) was reduced (p=0.002) at the second time point in the DYS group but not in the other groups.

Table 3. Total RNA concentration and RNA integrity of samples used for this research.

Cow #	Treatment	Week post calving	RNA concentration (ng/uL)	RIN
6048a	PPP	2	160	5.5
6048b	PPP	4	62.8	4.8
6057a	PPP	2	52.2	3.7
6057b	PPP	4	102	5.6
6071a	PPP	2	88.9	4.5
6071b	PPP	4	79.9	4.4
6015a	PRP	2	105	4.7
6015a	PRP	4	91.4	4.8
6019a	PRP	2	208	5.7
6019b	PRP	4	117	3.7
6041a	PRP	2	76.1	4.8
6041b	PRP	4	61.9	2.7
6018a	DYS	2	177	5.3
6018b	DYS	4	190	5.7
6031a	DYS	2	62.7	4.7
6031b	DYS	4	123	6
6052a	DYS	2	70.6	5
6052b	DYS	4	498	4.3
6017a	EUT	2	60.6	3.3
6017b	EUT	4	41.9	4.1
6027a	EUT	2	39.9	4.6
6027b	EUT	4	84.5	4.4
6046a	EUT	2	44.3	2.6
6046b	EUT	4	189	6.1





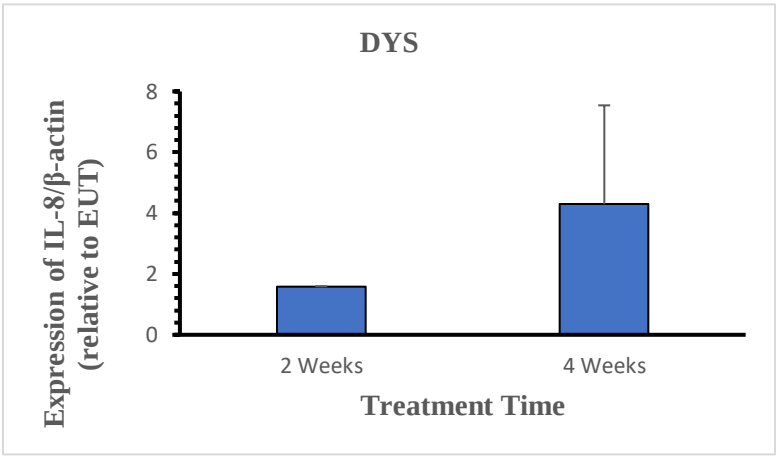


Figure 1. IL-8 relative gene expression in endometrial cells from normal-calving heifers treated with platelet-rich plasma (PRP) or platelet-poor plasma (PPP) or difficult-calving heifers treated with saline (DYS).

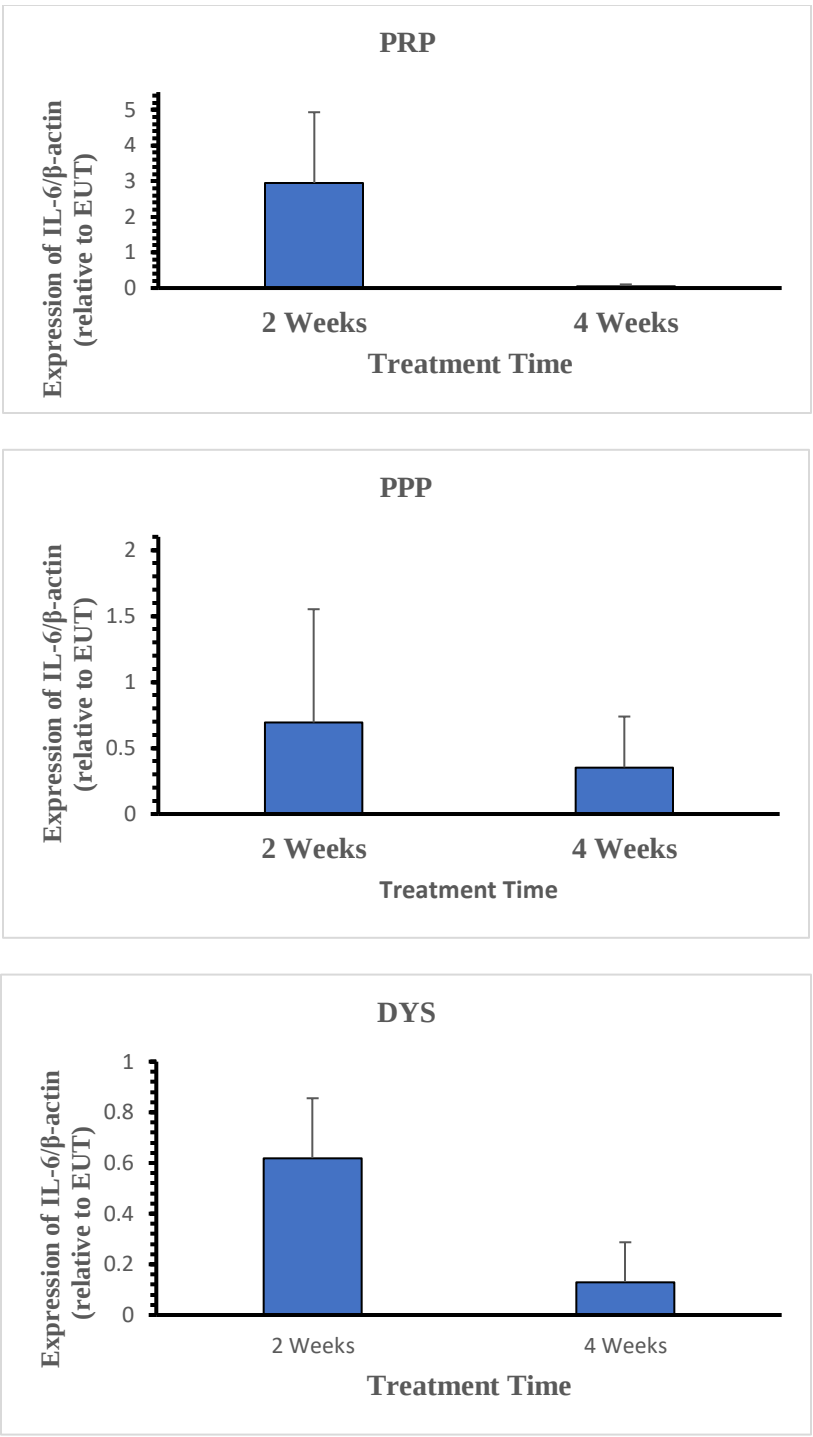
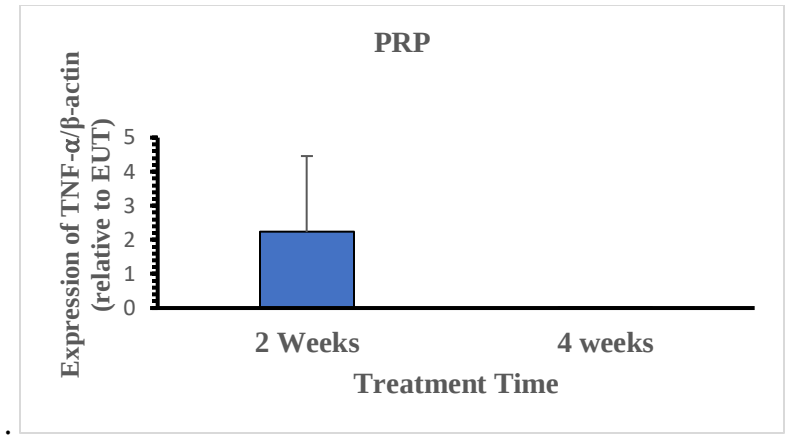


Figure 2. IL-6 relative gene expression in endometrial cells from normal-calving heifers treated with platelet-rich plasma (PRP) or platelet-poor plasma (PPP) or difficult-calving heifers treated with saline (DYS).



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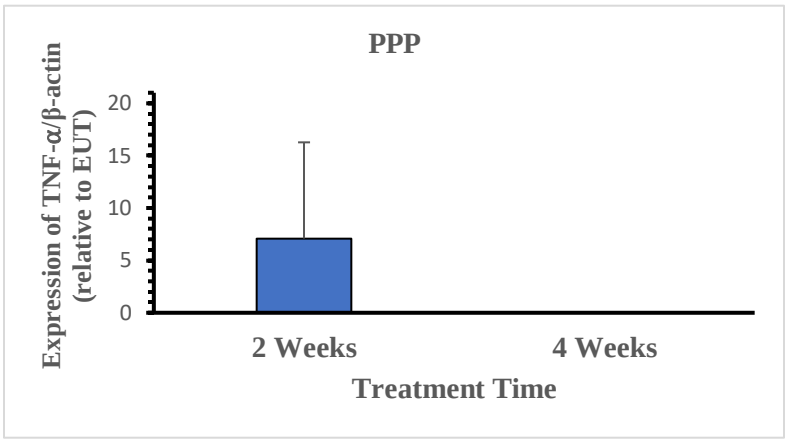

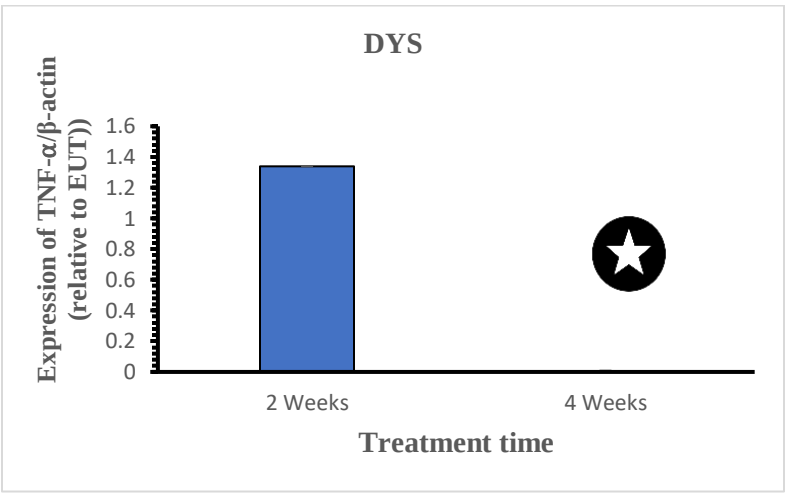

Figure 3. TNFα relative gene expression in endometrial cells from normal-calving heifers treated with platelet-rich plasma (PRP) or platelet-poor plasma (PPP) or difficult-calving heifers treated with saline (DYS). In DYS, TNFα expression was reduced (p = 0.002428).

Discussion

To our knowledge, this is the first report of the effects of PRP on gene expression of inflammatory cytokines in endometrial cells of beef cattle post-partum. This study could establish the foundation for alternative treatments for subclinical endometritis in beef heifers. Although, there are minimal studies on the expression of endometrial cytokines in beef heifers, our study may provide a groundwork to an alternative medication free from hormones and antibiotics. The results showed no difference between treatment groups. However, there was a decrease in TNFα gene expression in the SHD group from 2 weeks to 4 weeks post calving. Although these heifers did not present with any signs of uterine disease when samples were collected, higher expression of TNFα at 2 weeks post calving is consistent with subclinical endometritis[21].

Decreased expression of pro-inflammatory cytokines in the first week postpartum, leads to poor activation of neutrophils and monocytes, which impairs bacterial clearance and may cause cows to development endometritis[21]. According to Galvão and colleagues[21], healthy cows have a more robust pro-inflammatory response after calving, which allows complete endometrial healing more rapidly[22].

This research had certain limitations. The RIN for the samples was low although this did not appear to affect cDNA synthesis or quality. All of the samples were collected from healthy cows with no evidence of endometritis so it is not known how the treatment would affect cytokine expression in cows with endometritis.

Conclusion

Difficult calving increases the risk for developing endometritis but in healthy cattle, the increase in cytokine expression related to dystocia will resolve spontaneously. This explains why the relative expression of TNFα was higher 2-weeks post-calving compared to 4-weeks post-calving. This study also shows that intrauterine administration of platelet-rich plasma to normal calving heifers does not alter endometrial cytokine gene expression. Future studies will focus on investigating the effect of PRP treatment in cattle that experienced dystocia or have clinical evidence of endometritis.

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