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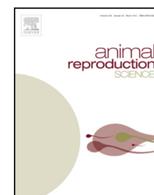

Endometrial expression of selected transcripts in postpartum of primiparous Holstein cows with clinical and subclinical endometritis

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ABSTRACT

Postpartum endometritis compromises milk production and fertility in high-producing dairy cows. Infection of the endometrium induces an inflammatory response with secretion of cytokines that lead to polymorphonuclear cells (PMN) influx and bacterial clearance. Considering that only a portion of cows with endometritis is eligible for clinical diagnosis, there is an increasing effort for developing reliable tools and protocols for diagnosis of subclinical endometritis. Recent reports have indicated that primiparous cows are at greater risk of uterine infection and primiparous cows with subclinical endometritis produce less milk compared to healthy cows. In the present study, gene expression profiles were compared for selected cytokine and hormone endometrial transcripts in the postpartum of primiparous Holstein cows with clinical and subclinical endometritis. Cows were classified as healthy (no signs of clinical endometritis), cows with subclinical endometritis (PMN < 5% in the cytological sample) and cows with clinical endometritis (PMN > 5%). Although, cows with clinical endometritis had greater ($P < 0.05$) relative amounts of mRNA for the *IL1A*, *IL6*, *IL17A*, *TNFα*, *PGES* and *PGHS2* genes compared to healthy cows; no significant differences were detected between clinical and subclinical endometritis groups. Spearman correlation coefficients were positive between relative amounts of gene expression as indicated by amount of these transcripts and PMN percentages and ranged from 0.74 to 0.93 ($P < 0.05$). Relative amounts of cytokine mRNA suggest similar inflammatory response in the endometrium of cows with subclinical and clinical endometritis. Moreover, differential relative amounts of hormone transcripts suggest dysregulation of the luteolytic mechanism and PG synthases but not ERα in cows with endometritis.

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1. Introduction

Postpartum endometritis caused by persistent bacterial infection is prevalent in high-producing dairy cows and leads to reduced milk yield and fertility (LeBlanc

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et al., 2002). Presence of pathogenic bacteria in the uterus causes inflammation, histological lesions of the endometrium, and perturbs uterine involution, ovulation and embryo survival (Sheldon et al., 2006). Whereas, clinical endometritis is associated with the presence of sufficient polymorphonuclear cells (PMN) influx to result in purulent or mucopurulent uterine exudates through the cervix; subclinical endometritis is characterized by abnormally larger numbers of PMN in the uterine lumen without clinical signs, including purulent material in the vagina (Gilbert et al., 2005). Bacterial infection of the endometrium induces an inflammatory response with secretion of chemokines and cytokines including tumor necrosis factor α (TNFα), interleukin 1A and 6 (IL1A and IL6) (Roach et al., 2002; Chapwanya et al., 2012). TNFα and IL1A stimulate gene expression for potent chemotactic factors (IL8, monocyte chemotactic protein-1, (MCP-1)), and adhesion molecules on vascular endothelial cells, leading to PMN recruitment to the site of inflammation (Sica et al., 1990; Roach et al., 2002). Similarly, greater expression of the IL6 gene has been detected in cows with endometritis and elevated amounts of IL6 in serum before parturition has been detected in cows susceptible for developing endometritis postpartum (Ishikawa et al., 2004; Galvao et al., 2011). Furthermore, interleukin17A (IL17A) is produced by the lymphocyte T helper 17 (Th17) cells and has been involved in host defence in epithelial and mucosal barriers against several pathogens (Jin and Dong, 2013). Its role in endometritis in cattle has not been characterized; however, IL17A has been described in various immune responses and inflammation conditions (Kolls and Linden, 2004).

The inflammatory response in the endometrium against bacterial infection is also mediated by pro-inflammatory molecules such as prostaglandins (PG), which have roles as multifunctional factors that regulate production of cytokines and mediate the luteolytic mechanism during the estrous cycle (Arosh et al., 2002). Prostaglandin G/H synthases (PGHS2) is an enzyme involved in the conversion of arachidonic acid into PGH2, a common precursor for various forms of PG including PGE2 and PGF2α. The downstream enzyme PGE synthase (PGES) catalyzes the conversion of PGH2 to PGE2, which regulates production of various cytokines including TNFα and IL6 and is also involved in maternal recognition of pregnancy (Bos et al., 2004; Arosh et al., 2002). PGF2α secretion from the endometrium is an important regulator of the estrous cycle as it initiates the regression of the corpus luteum (McCracken et al., 1999). Luteolysis in cows is preceded by activation of an estrogen receptor (ERα) which influences the timing of upregulation of endometrial oxytocin receptor and subsequently the release of PG (Robinson et al., 1999).

Elucidating the molecular mechanism controlling the local immune response is important for diagnosing and controlling postpartum uterine infection and potentially for identifying prognostic indicators for cows undergoing clinical and subclinical endometritis. The objective of the present study was to compare selected cytokine and hormone endometrial gene expression profiles in postpartum of primiparous Holstein cows with clinical and subclinical endometritis.

2. Materials and methods

2.1. Experimental animals

All procedures were approved by the Bioethical Committee of the Faculty of Veterinary Sciences at University of Chile (Certificate No. N21-2014). Cows on a large scale (~1000 cows) commercial dairy farm in the central zone of Chile were examined at days 29–36 postpartum (pp) by palpation of the uterus per rectum, manual examination of the vagina and vaginoscopy. Approximately, 38% of lactating cows were primiparous and from this group, 25% were diagnosed with endometritis by clinical examination. Three groups ($n = 6$ cows per each group) were defined. Group 1, included primiparous healthy cows with no signs of clinical endometritis (i.e. presence of purulent or mucopurulent vaginal discharge) and no signs of subclinical endometritis (PMN < 5% in the cytological sample; Gilbert et al., 2005). Group 2, consisted of primiparous cows with subclinical endometritis. These cows had no clinical signs for endometritis but the percentage of PMN in the cytological sample was >5. Group 3 consisted of primiparous cows with signs for clinical endometritis (i.e. purulent or mucopurulent vaginal discharge).

2.2. Cytological tests

For each animal, an endometrial epithelium sample was collected from the uterine horn using a trans-cervical guarded swab (Noakes et al., 1989). The swab comprised a long plastic rod bearing a cotton wool tip sheathed in a plastic guard tube. The guard tube was covered by sterile plastic sheath to prevent contamination of the swab during the cervix insertion. After restraining the animal and securing its tail, the perineal region was washed and cleaned. The cervix was grasped per-rectum and the sterilized catheter was passed through the cervix into the right uterine horn. The inner rod of the catheter was pushed forward to expose the swab to the endometrium and was rotated against the uterine wall and withdrawn within the catheter. Smears were prepared for cytological examination by rolling swabs on glass slides. The smears were then allowed to dry at room temperature for 30–35 min. Slides were transported to the laboratory and a differential cell count of each smear was done on Giemsa-stained slides. Cells ($n = 200$) were counted in each of 20 microscopic fields (900×).

2.3. Endometrial biopsy

Endometrial epithelium samples were collected from the uterine horn *ex vivo* for quantitative-PCR (Q-PCR) analyses using a Hauptner biopsy instrument (Kevorkian's uterine biopsy forceps) (Galvao et al., 2011). Briefly, after cytology sample collection, the biopsy instrument covered with a protective sheath was introduced into the vagina and guided into the cervix by manipulation *per rectum*. The instrument alone was introduced into the uterus after rupturing the sheath at the external cervical orifice and guided into the right horn past the uterine bifurcation. After collection, samples were immediately fixed in 500 μL of RNAlater (Qiagen Incorporated, Valencia, CA, USA) and

Table 1
Sequence of primers used for Q-PCR analysis.

Gene	Sense	Antisense	Accession number
Housekeeping			
GAPDH	5′ CCTTCATTGACCTTCACTACATGGTCTA	5′ TGGAAGATGGTGATGGCCTTTCCATTG	NM 001034034.2
SUZ12	5′ GAACACCTATCACACACATTCTTGT	5′ TAGAGGCGGTTGTGTCCACT	XM 582605
Cytokine			
IL1A	5′ AGAGGATTCTCAGCTTCCTGTG	5′ ATTTTTCTTGCTTTGTGGCAAT	NM 174092.1
IL6	5′ ATGACTTCTGCTTTCCCTACCC	5′ GCTGCTTTCACACTCATCATTC	NM 173923.2
IL17A	5′ TCCATCTCACAGCGAGCACAAG	5′ AGCCACCAGACTCAGAAGCAGTAG	NM 001008412.2
TNFα	5′ TCTTCTCAAGCCTCAAGTAACAAGT	5′ CCATGAGGGCATTGGCATAC	NM 173966.3
Hormone			
PGES	5′ GCGCGCTGCTGGTCATCAAA	5′ GTGTAGGCCAGGGAGCGGGT	NM 174443.2
PGHS2	5′ AGGTGTATGTATGAGTGTAGGA	5′ GTGCTGGGCAAAGAATGCAA	NM 174445.2
ERα	5′ ATGACCCTACCAGACCTTTCAGT	5′ ATTTGAGGCACACAAACTCTTC	NM 001001443.1

frozen at −80 °C until processing. One endometrial biopsy was performed from each animal for this experiment.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted using RNeasy Mini kit (Qiagen) according to the manufacturing's instructions. The concentration and purity of the RNA in each sample were determined using spectrophotometry (BioRad Laboratories, Hercules, CA, USA). Total RNA was eluted in 30–50 μL of RNase free water. Samples were subjected to RT-PCR using a Brilliant II SYBR Green RT-PCR kit (Agilent Technologies, Santa Clara, CA, USA). The reaction protocol consisted of incubation for 5 min at 25 °C, 15 min at 42 °C, 5 min at 95 °C and held at 4 °C using a DNA engine PCR thermocycler (Bio-Rad).

2.5. Quantitative-PCR

Real-time PCR primers were designed using PrimerExpress software (Applied Biosystems Incorporated, Foster City, CA) (Table 1). Real-time PCR primers were designed using PrimerExpress software (Applied Biosystems) (Table 1). Equivalence of amplification efficiencies among all primer-probe sets was confirmed using serial three-fold dilutions of differentiated MSC cDNA. Each RT-PCR reaction (25 μL) contained the following: 2× Brilliant II SYBR Green QPCR master mix (12.5 μL), diluted reference dye (0.375 μL), target forward primer (200 nM), target reverse primer (200 nM), cDNA synthesis reaction (2 μL) and nuclease-free PCR-grade water to adjust final volume. The PCR amplification was carried out in StepOne Real Time PCR System (Applied Biosystems). Thermal cycling conditions were 95 °C for 10 min, followed by 40 repetitive cycles at 95 °C for 30 s and 60 °C for 1 min. The PCR amplification was conducted in StepOne Real Time PCR System (Applied Biosystems). As a normalization control for RNA loading, parallel reactions in the same multiwell plate were performed using glyceraldehydes 3-phosphate dehydrogenase (*GAPDH*) and suppressor of zeste 12 (*SUZ12*) as a target. All reactions were performed in triplicate. In each experiment, amount of gene expression was recorded as CT values that corresponded to the number of cycles where the fluorescence signal can be detected above a threshold value. The CT averages for each biological replicate were calculated and transformed into relative values denominated quantity (*Q*) through ΔΔCT formula (Vandosempele et al., 2002). Then, the relative quantification in the expression of *IL1A*, *IL6*, *IL17A*, *TNFα*, *PGHS2*, *PGES*, *ERα* genes for each sample was estimated as the quotient between *Q* value of the target gene and a normalization factor (NF), which was calculated based on the geometric mean of housekeeping genes *Q* values (Vandosempele et al., 2002).

2.6. Data analyses

Values of gene expression from each group of cows were transferred to a spreadsheet and then analyzed using Infostat Software (Version 2013; National University of Cordoba, Argentina). Data were normalized to logarithmic scale in base 10 for normality and mean values were compared by one-way ANOVA. Gene expression values between groups of cows were analyzed using Duncan's multiple comparison test ($P < 0.05$).

3. Results

Average percentages of PMN were 3.03 ± 1.37; 13.67 ± 5.09 and 30.83 ± 6.07 in healthy cows and cows with subclinical and clinical endometritis, respectively (Fig. 1). The endometrium of cows with subclinical

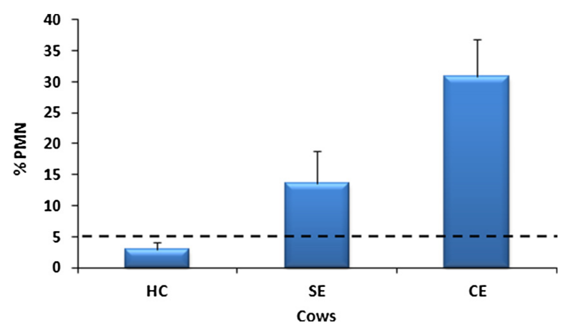

Fig. 1. Percentage of PMN in cytology samples collected during postpartum from healthy cows and cows with subclinical and clinical endometritis. Dashed line indicates cutoff value for diagnosis of subclinical endometritis (PMN = 5%) used in the present study; percentage of PMN were 3.03 ± 1.37 in healthy cows (HC), 13.67 ± 5.09 in cows with subclinical endometritis (SE) and 30.83 ± 6.07 in cows with clinical endometritis (CE).

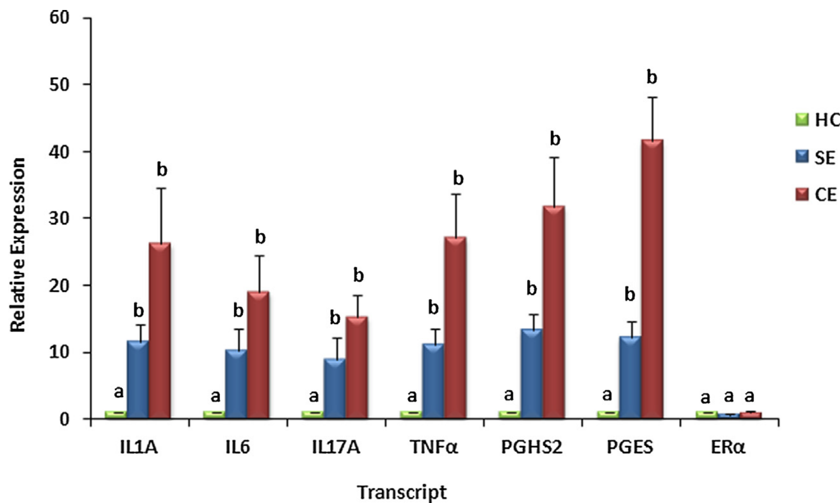

Fig. 2. Selected relative amounts of cytokine and hormone mRNA in endometrial tissue of primiparous Holstein cows with subclinical and clinical endometritis; cytokine and hormone relative amounts of gene expression were normalized using GAPDH and SUZ12 housekeeping genes; relative amounts of *IL1A*, *IL6*, *IL17A*, *TNFα*, *PGHS2* and *PGES* mRNA were greater ($P<0.05$) in cows with subclinical endometritis (SE) and clinical endometritis (CE) compared with healthy cows (HC); relative amounts of *ERα* mRNA were not different ($P>0.05$) between groups of cows; [a,b]different superscripts indicate differences ($P<0.05$) between groups of cows for each transcript.

endometritis and clinical endometritis had greater amounts ($P<0.05$) of mRNA for the *IL1A* (11.6- and 26.3-fold healthy cows), *IL6* (10.1- and 19-fold healthy cows), *IL17A* (8.9- and 15.34-fold healthy cows), *TNFα* (11- and 27.1-fold healthy cows), *PGHS2* (13.3- and 31.8-fold HC group), and *PGES* (12.2- and 41.5-fold healthy cows; Fig. 2). In contrast, relative amounts of mRNA for *ERα* gene were not different ($P>0.05$) between groups of cows (0.67- and 0.94-fold healthy cows). Spearman correlation coefficients were positive among relative amounts of *IL1A*, *IL6*, *IL17A*, *TNFα*, *PGHS2* and *PGES* mRNA and PMN percentages and ranged from 0.74 to 0.93 ($P<0.05$; Table 2). In contrast, coefficients among relative amounts of *ERα* mRNA and PMN percentages were not significant and ranged from −0.07 and 0.05.

4. Discussion

The early diagnosis and treatment of uterine infection is crucial to improve the reproductive performance of affected dairy cows. Considering that only a portion of cows with endometritis display visible discharge and are eligible for clinical diagnosis, there is an increasing effort for developing reliable tools and protocols for diagnosis of subclinical endometritis (Ghasemi et al., 2012). Recent reports have indicated that primiparous cows are at greater risk of uterine infection and primiparous cows with subclinical endometritis produce less milk compared with healthy cows (Galvao et al., 2010; Prunner et al., 2014). Therefore, an aim of the present study was to compare selected cytokine and hormone endometrial gene expression profiles during postpartum of primiparous Holstein cows with clinical and subclinical endometritis.

In the present study, up-regulation of the *IL1A*, *IL6* and *TNFα* genes as evidenced by relative amounts of mRNA transcripts in cows with subclinical and clinical endometritis suggested activation of pro-inflammatory cytokines involved in PMN and monocyte chemoattraction and promotion of phagocytosis. Although, cows with clinical endometritis had greater relative amounts of these transcripts compared to healthy cows, there were no significant differences detected between clinical and subclinical endometritis groups. Similar relative amounts of endometrial *IL1A*, *IL6* and *TNFα* mRNA in inflamed and healthy uteri have been reported in previous studies in cows sampled between 21 and 41 days

Table 2
Spearman correlation coefficients among relative amounts of cytokine and hormone mRNA and PMN percentage in endometrial tissue of postpartum healthy cows and cows with subclinical and clinical endometritis.

	IL1A	*IL6*	*IL17A*	*TNFα*	*PGHS2*	*PGES*	*ERα*	PMN
IL1A	–	0.91[a]	0.89[a]	0.87[a]	0.79[a]	0.83[a]	−0.07	0.91[a]
IL6	0.91[a]	–	0.93[a]	0.80[a]	0.75[a]	0.83[a]	−0.07	0.87[a]
IL17A	0.89[a]	0.93[a]	–	0.83[a]	0.84[a]	0.86[a]	−0.04	0.81[a]
TNFα	0.87[a]	0.80[a]	0.83[a]	–	0.87[a]	0.93[a]	−0.05	0.74[a]
PGHS2	0.79[a]	0.75[a]	0.84[a]	0.87[a]	–	0.90[a]	−0.05	0.68[a]
PGES	0.83[a]	0.83[a]	0.86[a]	0.93[a]	0.90[a]	–	0.01	0.75[a]
ERα	−0.07	−0.07	– 0.04	−0.05	−0.05	0.01	–	−0.25
PMN	0.91[a]	0.87[a]	0.81[a]	0.74[a]	0.68[a]	0.75[a]	−0.25	–

[a] Superscript indicate correlations ($P<0.05$) among variables.

postpartum (Gabler et al., 2009; Ghasemi et al., 2012). Differences in relative amounts of *TNFα* mRNA among cows with clinical and subclinical endometritis along with the findings that there were no differences in relative amounts of *IL6* mRNA have, however, been documented among healthy and diseased cows (Kasimanickam et al., 2014). Application of different cutoff for percentage of PMN for diagnosis of subclinical endometritis between studies (ranged from >5% to >18%) may explain differences in endometrial cytokine gene expression due to different amounts of inflammation in uterus and endometrium (Gabler et al., 2009; Galvao et al., 2011; Ghasemi et al., 2012; Kasimanickam et al., 2014). A recent study characterized the percentage of PMN in normal estrous cyclic cows throughout the cycle and described values of 8%, 6%, and 4% PMN for 21–33, 34–47, and 48–62 days postpartum in endometrial cytology of samples collected using the cytobrush technique (Madoz et al., 2013). These data support the hypothesis that percentage of PMN does not vary during the estrous cycle in normal cows and that a global cutoff of 5% PMN at 21–62 days postpartum may be used for diagnosis of subclinical endometritis.

Despite a reported role for IL17A in various immune responses and inflammation including arthritis, asthma and ulcerative colitis (Kolls and Linden, 2004); the participation of IL17A in endometritis has not been reported. IL17A is a key effector molecule of TH17 cells and promote granulopoesis, neutrophil accumulation, and neutrophil activation in the lung, joint space and intestinal tissue (Kolls and Linden, 2004). Moreover, IL17A exerts a synergistic effect with TNFα to induce secretion of IL8 and PGHS2 and proliferation of endometrial stromal cells (Hirata et al., 2010). In the present study, there was a similar pattern of endometrial expression of the *IL17A* gene as evidenced by greater amounts of mRNA transcripts compared with other cytokine transcripts evaluated in the postpartum of primiparous cows. Up-regulation of the *IL17* gene as evidenced by greater amounts of the corresponding mRNA transcript in cows with endometritis suggested the participation of this cytokine in uterine diseases of cattle (Jin and Dong, 2013). Moreover, the association that was detected between *IL17A* gene expression as evidenced by relative amount of the corresponding mRNA transcript and percentage of PMN supports a potential role in PMN recruitment and also for diagnostic purposes.

In ruminants, PGF2α and PGE2 are the primary PG produced in the endometrium with different secretory patterns and physiological effects. While endometrial PGF2α is secreted in a series of pulses inducing luteolysis, PGE2 is involved in maternal recognition of pregnancy and functions as a temporary luteotrophic signal in ruminants (Milvae et al., 1996; McCracken et al., 1999). Despite differential secretory patterns of PGF2α and PGE2, the ratio of PGE2/PGF2α during the luteal phase of the estrous cycle is considered more important than the absolute concentrations (Parent et al., 2002). PGHS2 and PGES are rate-limiting enzymes involved in the biosynthetic pathway for PGF2α and PGE2 (Jouzeau et al., 1997). In the present study, there was an up-regulation of relative amounts of mRNA for *PGES* and *PGHS2* in cows with clinical and subclinical endometritis which suggests its participation in pro-inflammatory events. These data support the hypothesis that inflammation of the uterus, as a consequence of pathogen infection, dysregulates PG biosynthetic pathway and may disturb the embryonic and luteolytic PG signaling, impairing reproductive performance (Gabler et al., 2009). The luteolytic mechanisms; however, may not be altered at the receptor for estradiol because relative amounts of ERα mRNA detected in the present study were not affected by uterine disease and were not correlated with PMN influx.

Selection of endogenous control gene to normalize gene expression data is an important consideration in the experimental design using Q-PCR analyses. Studies of endometrial gene expression in cattle have used a variety of endogenous genes including the transcripts for *GAPDH*, 18S ribosomal RNA (*18S rRNA*), β actin and bovine ribosomal protein (*BRP*) (Galvao et al., 2011; Gabler et al., 2009; Ghasemi et al., 2012; Kasimanickam et al., 2014). The most widely used endogenous gene in studies of endometrial gene expression is *GAPDH* (Walker et al., 2009). However, considering its potential regulation in a wide variety of physiological states, the suitability of *GAPDH* as an endogenous control gene has recently come into question (Olsvik et al., 2005). Based on comparison of gene stability between 15 candidate genes, a recent report indicated that *SUZ12* was the most appropriate control gene for use in endometrium of cattle during the estrous cycle (Walker et al., 2009). Thus, in the present study data were normalized using gene expression for both *SUZ12* and *GAPDH* as endogenous genes allowing for stability for relative gene expression analyses.

In conclusion, endometrial expression of selected cytokine genes including *IL1A*, *IL6*, *IL17A* and *TNFα* and PG enzymes *PGHS2* and *PGES* as indicated by relative amounts of corresponding mRNA transcripts were upregulated in postpartum of primiparous dairy cows undergoing subclinical and clinical endometritis. Relative amounts of cytokine mRNA suggest similar inflammatory response in the endometrium of primiparous cows with subclinical and clinical endometritis. Results suggest that the luteolytic mechanism may be dysregulated for PG synthases but not for ERα synthesis in cows with endometritis.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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