Differential expression of bovine major acute phase proteins, cytokines and metabolic indicator genes in clinical endometritis cows

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ABSTRACT

Among the uterine diseases, clinical endometritis (CE) is a major challenge to livestock farming as it causes sub- or infertility problems in dairy animals. The aim of this study was to evaluate the expression of cytokines (IL-1β, TNF-α, IL-6, and IL-8), acute phase proteins [APPs; haptoglobin (Hp), serum amyloid A (SAA) and alpha-1 acid glycoprotein (AGP)] and energy indicators [leptin and insulin-like growth factor (IGF)-1] genes in uterine tissue of CE affected cows. The uterine biopsy from CE cows (4) and non-endometritis cows (4) was processed for quantitative real-time PCR to study the mRNA expression of these innate immune molecules. We observed that mRNA expression of SAA, IL-1β, IL-8 and leptin genes were significantly up-regulated while, TNF-α and IGF-I genes were significantly down-regulated in CE cows. It can be concluded that bovine APPs, cytokines and energy indicators genes are differentially expressed in CE affected cows.

Key words: Acute phase proteins, Clinical endometritis, Cows, Energy indicators, Gene expression, Pro-inflammatory cytokines

Bacterial contamination of the uterine lumen is the common phenomenon during first week of postpartum in 90 to 100% of the dairy cattle (Williams et al. 2007, Sheldon et al. 2009). However, majority of the animals are effectively eliminating the bacteria and maintain optimal fertility, but 25 to 30% of animals still have persisted uterine infection which often leads to sub or fertile conditions (Gilbert et al. 1998, Sheldon et al. 2009). The bovine endometrium is well constructed with innate immune system to defense against invading bacteria after calving. The detector molecules such as Toll-like receptors at the endometrium recognize the microorganisms with help of pathogen-associated molecular patterns and subsequently alert the immune cells through mediators such as pro-inflammatory cytokines (Roach et al. 2002, Davies et al. 2008, Herath et al. 2006). Pro-inflammatory cytokines such as IL-6, TNF-α, and IL-8 stimulate the production of antimicrobial peptides by endometrial cells or accelerate the polymorphonuclear (PMN) cells infiltration into endometrium for elimination of pathogens. However, adequate stimulation of pro-inflammatory cytokines is critical for the healthy uterus as higher or lower expressions are often associated with greater inflammation or impaired chemotaxis, respectively during early postpartum period (Manimaran et al. 2016). Major bovine APPs (Hp and SAA) play an important role in the reproductive processes through intensification of the phagocytosis process against the uterine pathogens and reconstruction of the endometrium (Krakowski and Zdzisinska 2007). Therefore, efficient functions of detector, mediator and effectors molecules are important for elimination of uterine pathogens. Several researchers studied the level of APPs and cytokines mRNA transcription in endometrium and reported different results (Chapwanya et al. 2009, 2013; Lecchi et al. 2009, Gabler et al. 2010, Rahman et al. 2010). Among the various factors, the energy status of the peripartum animals is one of the most important determinants for the development of uterine disease and negative energy balance (NEB)-mediated alterations of gene expression have an important role in uterine immunity. For instance, Beam and Butler (1998) reported that severe NEB had increased uterine pro-inflammatory cytokine gene expression at two weeks postpartum and Wathes et al. (2009) reported that NEB caused more expression of uterine inflammation-associated genes. Fischer et al. (2010) reported that the higher expression of IL-1β, IL-8 and TNF-
α mRNA transcript in clinical endometritis (CE) cows while there was no correlation with uterine health for IL-6 and Hp transcripts. Collectively it indicated that the expression profile of innate immune molecules is differentially altered in the inflamed uterus and it needs further investigation. Hence the aim of this study was to evaluate the endometrial expression profile of bovine major acute phase proteins, cytokines and metabolic indicator genes in clinical endometritis affected cows.

MATERIALS AND METHODS

Experimental animals: The present study was carried out on Karan Fries (Holstein × Tharparkar) cows maintained at the Livestock Research Centre of the institute. The experimental procedures were duly approved by Institute Animal Ethical Committee (IAEC). The experimental animals were maintained in a loose housing system under group management practice. The space in the paddock, feeding manger and watering trough was as per the Bureau of Indian Standards. The nutrient requirements of the animals were mostly met with ad lib. green fodder, dry fodder, silage and a measured amount of concentrate.

Diagnosis and uterine biopsy sampling: Cows with purulent or mucopurulent uterine discharge during 22–47 days postpartum, were considered as suffering from clinical endometritis (CE) while, animals with clear mucus discharge were considered as clinically normal (non endometritis) (Sheldon et al. 2006). The uterine biopsy samples collected from CE affected and clinically normal cows (n=4 from each category) were processed for expression studies. All biopsies were performed by the same operator (veterinarian) and forceps were sterilized with isopropyl alcohol (70% v/v) swab before each sampling. Briefly, after cleaning the perineum and external genitalia, the biopsy instrument in a protective sheath (M/s IMV Technologies, France) was introduced into the vulval opening. The forceps was guided into the cervix with the left hand of the operator per-rectally covered by sterile latex glove. Then the instrument alone was introduced into the uterus after rupturing the sheath at the external cervical orifice and guided into the uterus. To ensure reproducible tissue (30–50 mg) procurement, with due care, the tip of the biopsy instrument inside the uterus was identified using the left hand per rectum. Then the forceps jaws were opened, and, with the help of left hand in the rectum, the endometrial tissue was clipped off by closing jaws and withdrawing the instrument. Tissue was immediately collected in sterile eppendorf tube containing RNA later (M/s Qiagen, Austin, USA) and then brought to the laboratory immediately and stored at –80°C till further processing. The biopsy materials were processed for quantitative real-time PCR to study the mRNA expression of APPs (Hp, SAA and AGP), cytokines (IL-1β, TNF-α, IL-6 and IL-8) and metabolites (IGF-1 and Leptin).

RNA isolation and cDNA synthesis: The RNA was isolated from 30 to 50 mg endometrial tissue stored in RNA later using Qiagen RNeasy mini kit (M/s Qiagen, Austin, USA) as per manufacturer’s protocol. The isolated RNA was treated with DNase as a cleaning purpose. The agarose gel (1%) electrophoresis was run to check quality of RNA samples while; concentration and purity of RNA samples were determined using NanoDrop microvolume spectrophotometer (M/s Thermo Scientific, Wilmington, USA). The single-stranded cDNA was synthesized using an RT-PCR kit (M/s Thermo Scientific, USA) as per standard procedure.

Quantitative real-time PCR: For mRNA quantification, the selected primer sequence was taken from the published literature and procured from M/s Sigma (USA). The sequence of the primer used in the present study, annealing temperature (Tm) used for real-time PCR and resulting size of the PCR amplified products are given in Table 1.

The fold change (n-fold) in gene expression was calculated using the relative quantitation method (2^-ΔΔCt) having β-actin as the endogenous control and the average ΔCt for samples collected from healthy animal as the calibrator for each sample. ΔCt values from the Ct values were calculated by subtracting β-actin Ct values from the Ct values of a specific target gene for all cows (ΔCt normalized target gene=target gene Ct – β-actin Ct). Then, ΔΔCt values were calculated by subtracting the average ΔCt normalized for all healthy cows (calibrator) from ΔCt normalized target (ΔΔCt=ΔCt normalized target –ΔCt normalised calibrator), then, n-fold (2^-ΔΔCt) calculated. Therefore, the n-fold represents the endometrial gene expression in all endometritis cows in relation to healthy cows, normalized to endogenous control (β-actin). Initially, for endogenous control, two genes, viz. GAPDH and β-actin were taken and β-actin was selected for endogenous control as its expression was similar for endometritis cows (21.2–22.6) and for control cows (21.3–23.8). In the present study, data are presented as the changes in threshold cycle (ΔCt) value relative to the house-keeping gene, β-actin.

RESULTS AND DISCUSSION

It is known that gene expression levels differ in various cell types and during various clinical conditions. However, how the gene expression is altered during various pathophysiological conditions is largely unknown. Although mRNA is not the ultimate product of a gene, transcription is the first step in gene regulation and therefore information at the transcription level is important for understanding gene regulation. In the present study, IL-1β and IL-8 expression were significantly (P<0.05) up-regulated about 3 and 2.5 folds respectively, in the CE cows. Fischer et al. (2010) found significantly higher expression of IL-1β and IL-8 mRNA in cows affected with subclinical or CE, compared with healthy cows. Ghasemi et al. (2012) found a higher mRNA expression of IL-8 level in subclinical endometritis cows and suggested that IL-8 gene expression may be useful to predict endometrial inflammation. Higher expression of pro-inflammatory cytokines (IL-1β, IL-6, and IL-8) was also reported by several researchers (Ishikawa et al. 2004, Fischer et al. 2010, Gabler et al. 2010, Loyi et al. 2013,
Swangchan-Uthai et al. 2012, Chapwanya et al. 2013). In the present study, IL-6 was nonsignificantly up-regulated, while TNF-α expression was nonsignificantly down-regulated in the endometritic cows. Several researchers (Nino-Soto et al. 2008, Chapwanya et al. 2009, Fischer et al. 2010, Galvao et al. 2011, 2012) also found that cows affected with metritis and endometritis had lower expression of TNF-α than the healthy counterparts. TNF-α is the main cytokine involved in the stimulation of the expression of adhesion molecules, such as E-selection, which in turn play an essential role in the recruitment of polymorphonuclear cells (PMNs) in response to IL-8 (Roach et al. 2002). In the present study, decreased TNF-α gene expression could have led to poor chemotaxis and lesser activation of PMNs which resulted in delayed bacterial clearance and development of endometritis (Galvao et al. 2011). Further, the lower expression of TNF-α could also have facilitated for CL maintenance (Okuda and Sakumoto 2003). Collectively, it indicated that the pro-inflammatory cytokine expressions are altered during endometritis and thus it compromised the ability of cows to respond to bacterial contamination during the postpartum period.

Hp mRNA expression was up-regulated nonsignificantly (1.65 folds), while SAA mRNA expression was up-regulated (63 folds) significantly (P<0.01) in the CE cows. Gabler et al. (2010) reported that Hp mRNA content did not differ between inflamed and healthy endometrium and found low abundance of protein in bovine uterus. Fischer et al. (2010) also reported that Hp transcripts were not correlated with uterine health conditions. Chapwanya et al. (2013) also reported that the SAA mRNA expression was higher than Hp and other cytokines mRNA expression as observed in our study. Regulation of Hp expression is complex and it is both tissue and species-specific (Pajovic et al. 1994). For example, maximal Hp expression requires glucocorticoids and IL-6 in human hepatic cells (Baumann et al. 1990). The lesser expression of IL-6 may substantiate the absence of stronger stimulation and thus low expression of Hp (Mueller-Doblies et al. 2004). Quantitative PCR and immunohistochemistry studies indicated minimal AGP expression in clinically healthy or infected bovine uterus (Lecchi et al. 2009), as observed in this study.

The altered immune response could be due to an intrinsic defect in endometrial cell function or extrinsic mechanisms affecting endometrial cell activity such as a negative energy balance (NEB). Beam and Butler (1998) reported that differences in cytokine gene expression between CE and healthy cows were more prominent under influence of NEB. Before development of CE in these cows, higher levels of non-esterified fatty acids (NEFA) and beta hydroxy butyric acid (BHBA) were observed during transition period (data not presented). It clearly indicated the presence of NEB preceding to CE development in these cows. Therefore, NEB during early postpartum might have compromised uterine immunity and thus facilitated the development of CE during late postpartum. In the present study, leptin mRNA expression was significantly (P<0.05) up-regulated (7.9 fold), while IGF-1 expression was significantly (P<0.05) down-regulated (4.28 fold) in the CE cows. We also found significantly (P<0.05) higher concentration of leptin during transition period in these cows before development of CE (data not presented). Kasimanickam et al. (2013) also reported higher leptin levels in the CE cows. During acute inflammation, increase in peripheral

### Table 1. Primers used for gene-specific RT-PCR and real-time PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Tm</th>
<th>Size (bp)</th>
<th>Accession No.</th>
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<tr>
<td>Bov IL6-F</td>
<td>CGAGGACGAGCAAGACG</td>
<td>60.7</td>
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<td>CAGAGTCAACACGAGAG</td>
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<td>Bov IL8-F</td>
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<tr>
<td>Bov TNFα-F</td>
<td>CTTTCTGCTGCTGCACTTTC</td>
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<td>NM_173966.3</td>
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<td>Bov TNFα-R</td>
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<tr>
<td>Bov GAPDH-F</td>
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<td>Bov SAA3-R</td>
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<td>Bov Leptin-F</td>
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cytokines concentration is associated with increase in tissue cytokine expression (Kasimanickam et al. 2013). The influence of elevated leptin concentrations during transition period on endometrial expression of leptin gene during later postpartum period in relation to clinical endometritis in dairy cows cannot be ruled out. Similarly, CE affected cows had significantly down regulated IGF-I mRNA transcripts in bovine uterus along with lesser concentration of IGF-I during transition period. It was also suggested that NEB is an important determinant of IGF-I expression (Wathes et al. 2011). Therefore, the observed NEB during early postpartum in these cows may be related to lesser IGF-I transcripts levels in CE cows. Taken together, it is concluded that bovine acute phase proteins, inflammatory cytokines, and energy indicators genes are differentially expressed in clinical endometritis affected cows. The therapeutic use of this knowledge further needs to be investigated.

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