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Profiling inflammatory biomarkers in cervico-vaginal mucus (CVM) postpartum: Potential early indicators of bovine clinical endometritis?

Mounir Adnane [a, b, c], Aspinas Chapwanya [e], Rachid Kaidi [d], Kieran G. Meade [f, 1], Cliona O'Farrelly [a, *, 1]

[a] Comparative Immunology Group, School of Biochemistry and Immunology, Trinity College, Dublin, Ireland
[b] High National Veterinary School, Algiers, Algeria
[c] Institute of Veterinary Sciences, Tiaret, Algeria
[d] Institute of Veterinary Sciences, Blida, Algeria
[e] Ross University School of Veterinary Medicine, Basseterre, Saint Kitts and Nevis
[f] Animal & Bioscience Research Department, Animal & Grassland Research and Innovation Centre, Teagasc, Grange, Co. Meath, Ireland

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ABSTRACT

Endometritis significantly impacts fertility and milk yield, thus reducing profitability of the dairy production. In cows that develop endometritis, normal postpartum endometrial inflammation is dysregulated. Here, we propose that endometrial inflammation is reflected in cervico-vaginal mucus (CVM) which could therefore be used as a prognostic tool. CVM was collected from 20 dairy cows (10 with clinical endometritis and 10 healthy) 7 and 21 days postpartum (DPP). Polymorphonuclear (PMN), mononuclear leukocyte and epithelial cells were counted, total protein levels were estimated and levels of IL-1β, IL-6, IL-8, serum amyloid A (SAA), haptoglobin (Hp) and C5b were analyzed by ELISA in CVM. PMN were consistently high in CVM from 7 to 21 DPP, but were higher in CVM from cows with clinical endometritis 21 DPP compared with healthy cows. In contrast, there were more epithelial cells in healthy cows 21 DPP than in clinical endometritis animals. Total protein levels decreased significantly in CVM from healthy cows between days 7 and 21 postpartum. All inflammatory biomarkers except C5b, remained high in cows with clinical endometritis from 7 to 21 DPP, indicating sustained and chronic endometrial inflammation. IL1, IL-6, IL-8 and Hp levels were higher in CVM from cows with clinical endometritis compared to healthy cows 21 DPP. Interestingly IL-1β levels were raised in CVM from clinical endometritis but not in healthy cows 7 DPP suggesting that early measurement of IL-1β levels might provide a useful predictive marker of clinical endometritis. In contrast, SAA and C5b levels were increased in healthy cows 21 DPP, compared to cows with clinical endometritis suggesting that these acute phase proteins might have an anti-inflammatory role. Our results show that CVM is convenient for profiling disease-associated changes in key inflammatory molecules postpartum and reaffirms that sustained inflammation is a key feature of clinical endometritis in the dairy cow.

1. Introduction

The global incidence of uterine disease in cattle is variable [1–3] with particularly high incidences in high yielding dairy cows [4]. Postpartum uterine disease and inflammation compromise fertility by lengthening the calving interval, increasing number of services per conception and reducing calving rates [5–7]. Furthermore, dairy cows with endometritis spend less time at peak lactation and require frequent treatment [6,8,9]. Even after recovering from endometritis, fertility remains suboptimal [10]. Poor fertility, low milk production and the direct cost of treatment all result in significant loses to the dairy industry [11].

Normal activation of inflammation in the uterus starts pre-calving, in preparation for parturition, and escalates postpartum [12,13]. This healthy inflammation is crucial for evacuation of the fetus, fetal fluids, lochia and tissue debris. It also enables the

* Corresponding author. Comparative Immunology Research Group, Trinity Biomedical Sciences Institute (TBSI), 152-160 Pearse Street, Trinity College, Dublin, Ireland.
E-mail address: adnanem@tcd.ie (C. O'Farrelly).
[1] Both Kieran and Cliona contributed equally to the direction of this study.

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important events of caruncular shrinkage and endometrial tissue remodeling postpartum during healthy physiological uterine involution [12,14]. Healthy inflammation during normal involution, is characterized by polymorphonuclear leukocyte infiltration and elevated levels of proinflammatory cytokines and acute phase proteins which revert to normal levels two to three weeks postpartum [13,15]. But in cows which develop clinical endometritis, local endometrial inflammation persists [3,16]. A major challenge is to identify as early as possible the precise molecular mechanisms that regulate normal inflammation in healthy animals and those which signal pathological inflammation in cattle susceptible to clinical endometritis. Early identification of the key switch would allow farmers and veterinarians to predict disease-onset and administer appropriate prophylactic therapy.

Clinical endometritis is defined as a persistent inflammation of the endometrium 21 days postpartum (DPP), associated with mucopurulent or purulent vaginal discharge [17]. Over-stimulation by an altered microbiome and/or heightened host sensitivity to locally generated damage-associated molecular patterns (DAMPS) and microbe-associated molecular patterns (MAMPS) is likely to be responsible for the dysregulated local inflammatory response that characterizes endometritis [18]. Previously, we and others reported increased expression of innate immune genes including IL-1, IL-6, IL-8, serum amyloid A (SAA) and haptoglobin (Hp) in endometrial tissue from healthy cows after calving that returned to normal physiological levels 30 DPP [3,19,20]. Even higher levels of many of these inflammatory markers have been described in uterine tissue of cows with endometritis [18]. Next-generation sequencing of uterine biopsies from cows with cytological endometritis confirmed increased expression of many inflammation-related genes. Intriguingly, 7 DPP, significant differences in gene signatures were observed in animals with cytological endometritis when compared with healthy animals [18] suggesting that a disease-associated trajectory might be predicted as early as seven days after calving.

Most previous studies of inflammation in the uterus postpartum have focused on uterine tissue [3] or uterine washings [16,21,22] which can only be acquired from relatively few animals because of the time and technical expertise required. Clinical endometritis, characterized by purulent, mucopurulent or fetid vaginal discharge is currently diagnosed by either vaginoscopy, Metricheck or a gloved hand [15,23–25]. Cervico-vaginal mucus (CVM) which can be easily collected at any stage and does not require specialist training might therefore provide a more accessible resource to assess disease status. In addition to mucins and carbohydrates, CVM contains cytokines and other innate immune molecules which can be assayed [26–28]. Here, we hypothesize that the inflammatory signature of CVM mirrors inflammatory activity in the endometrial environment and may provide a useful source of biomarkers for clinical endometritis in dairy cows. With this in mind, we examined postpartum CVM collected 7 and 21 DPP for levels of candidate inflammatory markers including IL-1β, IL-6, IL-8, SAA, Hp and C5b.

2. Materials and methods

For this study, we examined 29 Holstein Friesian (HF) dairy cows in the first week of lactation. We selected 20 cows on the basis of not experiencing calving difficulties or showing any signs of ill-health during the postpartum period. The cows selected were aged 4–9 years (average = 5 years) in the second to eighth lactation. The clinical history; age, parity, calving ease were recorded on all animals. No parturition complications, retained placentae or metabolic diseases were diagnosed in the cows enrolled in this study.

Samples were collected under license number (AE 19132/P038) issued from the Health Products Regulatory Authority in Ireland.

2.1. CVM collection

To perform vaginal examination and mucus collection, using examination sleeves, the tail was lifted and the perineum inspected. The perineum was wiped with dry paper, or with 70% ethanol if necessary to remove fecal material. AT 7 DPP, a gloved hand was inserted through the vulva into the vagina. The hand was cupped and mucus collected and transferred into a test tube. At 21 DPP, CVM was collected using Metricheck™ (Simcro Limited, Hamilton, New Zealand) as previously described [25]. Briefly, the Metricheck device and cup was disinfected with 70% ethanol and gently introduced into the anterior vagina. Metricheck was then withdrawn and CVM collected in cups was scored and stored in 20 ml sterile tubes. A new examination cup was used for each cow. All CVM were scored using a previously described scale where score 2 and 3 at 21 DPP indicates endometritis [23]. CVM samples were transported to the laboratory on ice within 4–6 h of collection.

2.2. Cytological smear preparation

Duplicate cytology smears were prepared (Sterlin Ltd, Thermo Fisher Scientific, UK). The slides were air-dried and stained using Diff-Quick staining protocol (Speed-Diff, Clin-Tech Limited, Essex, UK). Smears were evaluated microscopically (magnification ×400) and proportions of neutrophils, macrophages, lymphocytes, basophils, eosinophils and epithelial cells were recorded.

2.3. CVM processing

In the laboratory, mucus was prepared according to the technique described for human sputum analysis [29] and adapted by Healy et al. [30] for analysis of CVM, with minor modifications, and stored at −80 °C until further analysis.

2.4. Measurement of total protein

Total protein level in CVM samples were determined using the bicinchoninic acid assay (BCA assay) (#23227, Pierce™ BCA Protein Assay Kit. Thermo Fisher Scientific Inc, UK) according to the manufacturer's instructions. Briefly, samples were thawed and centrifuged at 3000 × g for 15 min at 4 °C and 25 μl aliquots of the supernatant measured in duplicate in 96 well plates. Plates were incubated for 30 s with moderate shake and then incubated at 37 °C for 30 min. Optical density of each well was measured using BCA preconfigured protocol on the microplate reader.

2.5. Measurement of cytokines, APPs and complement component in CVM

Frozen CVM was thawed and centrifuged at 3000 × g for 15 min at 4 °C and supernatants was used to run ELISA. Levels of IL-1β, IL-6, IL-8, SAA, Hp and C5b were measured (IL-1β: #ESS0027 and IL-6: #ESS002 from Thermo Fisher Scientific Inc, UK; SAA: #TP-802 PHASE™ Serum Amyloid A Assay (SAA) kit and Hp: Tridelta PHASE™ Haptoglobin Assay kit, from Tridelta Development Ltd, Maynooth, Co. Kildare, Ireland and C5b: #MBS008530, MyBioSource, Inc, San Diego, CA 92195-3308, USA). Procedures were performed according to the guidelines provided by the manufacturer except for Il-8. IL-8 was measured using a sandwich ELISA optimized by Cronin et al. [28]. This technique was shown to be more sensitive for measurement of bovine IL-8 than the human IL-8 ELISA kit. To measure IL-8 in CVM, a monoclonal mouse anti-sheep