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Short communication

Alterations in cortisol concentrations and expression of certain genes associated with neutrophil functions in cows developing retention of fetal membranes

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ABSTRACT

Elevated cortisol concentrations have been reported to impair the functions and alter the life span of neutrophils in cows. The present study assessed the cortisol concentrations and expression of few genes related to longevity (Fas, Caspase 3, Bcl2) and margination (CD 62L, CD 18/11b) of neutrophils in relation to retention of fetal membranes (RFM) in dairy cows. Cortisol concentrations were significantly higher on the day of calving and day 2 postpartum in RFM cows than normal cows. Expression of CD 62L was significantly lower on the day of calving and day 2 postpartum in RFM cows than normal cows. While expression of Fas and GR was significantly lower on the day of calving, expression of Bcl2 was lower on day 7 ± 2 pre-partum in RFM cows compared to normal cows. No significant difference was observed in the expression of CD 18/11b and Caspase 3 between RFM and normal cows. Cortisol concentration was negatively correlated with expression of GR, Fas, CD 62L, CD18/CD11b and Caspase 3, while positively correlated with immature neutrophil percentage and expression of Bcl2. It may be inferred that cortisol concentrations and expression of certain genes associated with lifespan and margination of neutrophils were altered in cows developing RFM compared to those expelled the fetal membranes normally.

1. Introduction

Retention of fetal membranes (RFM), a condition in which the fetal membranes are not expelled within 12 h after parturition, leads to huge economic losses to the dairy industry (Beagley et al., 2010) since the production and reproduction potential of the affected cows are seriously compromised. It has been observed that the incidence of RFM in crossbred cattle was about 26%, comparatively higher than zebu cattle (16%) and buffaloes (13%; Kumari et al., 2015). In RFM affected cows, it has been reported that the average milk loss was to the tune of 753 kg per lactation (Duboc et al., 2011). Although the losses associated with RFM are well quantified, the etiology has not been understood in detail. In dairy cattle, it has been shown that RFM is associated with impaired neutrophil function and reduced chemotaxis (Kimura et al., 2002; Beagley et al., 2010). Recently it has been shown that cows affected with RFM had reduced concentrations of certain pro-inflammatory cytokines (Streyl et al., 2012; Boro et al., 2014, 2015).

Migration of neutrophils to the site of feto-maternal junction and its normal functioning is important for fetal membrane separation (Kimura et al., 2002; McNaughton and Murray, 2009). It has been reported that CD 62L and CD 18/11b regulate the margination (Weber et al., 2001, 2004; Burton et al., 2005) while Fas and Caspase 3 regulate the lifespan of neutrophils and any alterations in their expression may affect the normal functioning of the cell (Chang et al., 2004; Burton et al., 2005). Hormonal milieu of the blood and extracellular tissue fluid can influence neutrophil development and immunity related activities since the immune system is controlled by the endocrine system of the body (Bouman et al., 2005). Elevated concentrations of cortisol during parturition have an immunosuppressive effect and impair the neutrophil functions by affecting the activity and life pattern of neutrophils (Burton et al., 2005). Earlier studies have reported significantly higher concentrations of cortisol in cows developing RFM compared to the cows those expelled the fetal membranes normally (Gupta et al., 2004; Kaczmarowski et al., 2006; Beagley et al., 2010; Hanafi et al., 2011). Mature neutrophils respond to the high cortisol concentrations as they possess

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the glucocorticoid receptors (GRα) leading to altered neutrophil functioning and signaling. Further, due to alteration in the expression of some genes related to margination (CD 62L and CD 18/11b) and cell death (Fas, Caspase 3 and Bcl2), a condition of neutrophilia with an increased proportion of long lived neutrophil exists around the time of parturition (Burton et al., 2005). However, the molecular basis of these phenotypic changes and physiological benefits or drawbacks especially in relation to separation and expulsion of fetal membranes is poorly understood.

We hypothesized that alterations in the concentrations of cortisol and expression of genes associated with margination and apoptosis of neutrophil, contributing to an altered neutrophil function, may be associated with RFM in dairy cows. Thus the aim of the present study was to estimate peripheral concentrations of cortisol, expression of GR, Fas, CD 62L, CD 18/11b, Caspase 3 and Bcl2 in neutrophils from cows that developed RFM and cows that expelled fetal membranes normally.

2. Materials and methods

2.1. Selection of experimental cows and their management

The present study was conducted on advanced pregnant crossbred cows (Holstein Friesian × Tharparkar) maintained at Livestock Research Center, National Dairy Research Institute, Karnal, Haryana, India. Initially, a total of 33 healthy advanced pregnant cows (2nd–5th parity, 420–500 kg body weight, normal body condition and yielded 4000–4500 kg milk in previous lactation) were recruited for the study. All the cows were vaccinated against common diseases (like Brucellosis, Foot and Mouth Diseases, Hemorrhagic Septicemia and Black Quarter) as per the standard management practices of the farm. All the experimental procedures were approved by the institutional animal ethics committee (IRC project code B-25).

Cows were shifted from dry pregnant cow paddocks (brick on edge flooring) to the calving pen 1 week prior to expected date of calving and retained till 4–5 days after calving. Daily ration of the experimental cows consisted *ad libitum* green fodder (Maize, Jowar, Cowpea, Berseem and Oat) and measured amount of concentrates (20% crude protein and 70% total digestible nutrient). The cows were fed with concentrate mixture at 2.5–3.0 kg per/cow/day from 2–3 weeks prior to expected date of calving. The animals were fed as per National Research Council (2001) recommendation.

All the experimental cows were observed for expulsion of fetal membranes. Cows that expelled the fetal membranes within 12 h after delivery of the calf were grouped under normal parturition (NP) group while the cows that failed to expel the fetal membranes within 12 h after the delivery of calf were grouped under RFM group. Cows that had not expelled fetal membranes within 12 h after calving were examined per vaginal to confirm the presence of fetal membranes. Cows with abnormal calving like dystocia were excluded from the study. After eliminating the cows that calved 3 days or earlier than the expected date of calving and cows that had abnormal calvings, we selected six RFM cows and six NP cows for estimation of cortisol and gene expression studies.

2.2. Collection of blood samples and estimation of cortisol

Blood samples were collected from all the experimental cows during early morning between 7.00 A.M. and 8.00 A.M. on 7 ± 2 day prepartum, on the day of calving and day 2 postpartum using 9 ml vaccutainer (Vacutte®, Griner Bio-one Gmbh, Austria). Blood plasma was separated and stored at −20 °C for estimation of cortisol. Plasma cortisol levels were estimated by ELISA (Endocrine Technologies, USA Catalog- #ERK B1004) method. The minimum detectible concentration of cortisol by this assay was 0.1 ng/ml. The intra-assay variation was 5.7% and the inter-assay variation was 8.5%.

2.3. Blood neutrophil isolation and gene expression analysis

Neutrophil percent in blood was estimated by the differential leukocyte count as per the standard procedure using Leishman's stain. Total of 100 white blood cells were counted from each smear and the neutrophil percentage was calculated. Immature neutrophils count was estimated by using field staining. Total of 100 neutrophils were counted from each smear and immature neutrophil percentage was calculated.

Isolation of neutrophil from peripheral blood was performed using hypotonic lysis of erythrocytes as described by Mehrzad et al. (2001, 2004) and the isolated neutrophil was used for further analysis. DEPC-treated RNase free plastic wares and water was used for gene expression study. Trizol method was used for RNA extraction from the blood neutrophil (Chonczynski and Sacchi, 1987). The RNA pellet was air dried for 15–30 min and dissolved in 25 μl of RNA storage solution and stored at −80 °C till further use. Quality of RNA was checked by agarose gel electrophoresis using 1.5% gel (in 1× TAE buffer, pH 8.0) of high-quality molecular biology grade agarose (Sigma, USA). Ethidium bromide (at 0.5 μg/ml of gel) was used as fluorescence dye while bromophenol Blue (at 3 μl) as tracking dye which was mixed with RNA during time of loading of sample into well of the gel. Electrophoresis was carried out at 8 V/cm for around 30 min and examined under UV transilluminator. DNase treatment was done by using DNA free Kit (Ambion, UK). RNA was quantified and purity of RNA was judged on the basis of optical density ratio at 260:280 nm determined with ND-3300 flurospectrophotomer (NanoDrop Technologies, UT). Reverse transcription was performed from 1 μg of RNA using Novagen first strand cDNA synthesis kit. Real-time PCR for GR, Fas, CD 62L, CD 18/11b, Caspase 3 and Bcl2 was carried out using Roche Light Cycler-480 (Germany). The sequence information of gene was retrieved from NCBI database and suitable primers were designed using primer-3 web interfaces. Details of primers are given in the Table 1. Broadly for each real-time quantitative PCR (qPCR), 1 μg cDNA was added to a 20 μl mix containing primers, IQ SYBER-green supermix (Bio-Rad, USA) and nuclease free water. PCR conditions were 300 s at 95 °C, 45 cycles of 20 s at 95 °C, 20 s at appropriate annealing temperature and 20 s at 72 °C. A melting curve for each qPCR with a single peak at the correct melting temperature was indicative of reliable and desired PCR product. The mRNA abundance of NP cows was taken as calibrator with which relative expression of specific genes in RFM cows was estimated. Calculation was done using the 2-ΔΔCt method (Livak and Schmittgen, 2001). The house keeping genes used in the study as internal control were Bovine beta actin and bovine GAPDH.

2.4. Statistical analysis

Data sets were first analyzed for Normality test (Shapiro–Wilk test) and then general linear model was used to study the effects of group (RFM and NP) and period (day's effect) and their interaction. All pair-wise comparison of means were done by Tukey *post hoc* test and considered as significant at $P < 0.05$. The results were presented as mean ± SEM. Correlation analysis of different parameters was done by Pearson correlation technique. All the statistical analyses were carried out using SigmPlot 11® software package (Systat software Inc., San Jose, CA, USA).