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Short communication

Decreased cyclooxygenase-2 gene expression and lactoferrin release in blood neutrophils of heifers during the calving period

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ABSTRACT

Immunosuppression during the calving period in dairy cows is associated with an increased risk of diseases. Correct neutrophil function is a key mechanism of innate immunity that is used to protect the host from pathogenic microorganisms. The aim of this study was to evaluate the function of blood neutrophils obtained from heifers between 30 days preparturition and 30 days postparturition. We assessed the phagocytosis of fluorescent bioparticles using flow cytometry, chemotaxis induced by chemoattractants using the transwell plate assay, lactoferrin release using ELISA and cyclooxygenase-2 (COX-2) gene expression using real time-PCR. Our results showed an increased ability of phagocytosis of bioparticles and chemotaxis induced by the chemotactic agent platelet activating factor (PAF), between day 15 preparturition until day 30 postparturition, and at calving, respectively. COX-2 gene expression induced by PAF was increased only in neutrophils obtained at days 30 pre- and post-parturition ($p < 0.001$). Neutrophil lactoferrin release was reduced between day 15 preparturition and day 30 postparturition compared with that at day 30 preparturition. Furthermore, lactoferrin plasma levels were increased at calving. In conclusion, we provided evidence that neutrophils from heifers around calving time exhibit impairment of particular defensive functions, such as COX-2 mRNA expression and lactoferrin, suggesting that these mechanisms may contribute to immunosuppression in cows around calving.

1. Introduction

The transition period, which is 3 weeks before to 3 weeks after parturition, is characterised by important changes in metabolism and the host defence mechanism, which have been associated with an increased risk of diseases (Ingvartsen and Moyes, 2013). In this period, dairy cows enter a negative energy balance (NEB) because of increases in energy requirements due to foetal needs and lactogenesis and decreases in dry matter intake (Grummer et al., 2004). To adapt to this NEB, cows mobilise adipose energy depots, resulting in an increase in plasma non-esterified fatty acids (NEFA) and ketone bodies. NEFA have been suggested as a potential risk factor of diseases, such as mastitis (Melendez et al., 2009; Moyes et al., 2009), and can modify immune function in the transition dairy cow.

Abbreviations: COX-2, cyclooxygenase-2; PAF, platelet activating factor; NEFA, non-esterified fatty acids.

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Neutrophils are the first line of defence of the host against invasive microorganisms and are one of the first cells to migrate from the bloodstream into injured or infected tissues *via* chemotaxis (Smith, 1994). Neutrophils defend against engulfing pathogens and phagocytosis in tissues, releasing degradative enzymes and bacterial proteins stored in cytoplasmic granules, such as lactoferrin, and produce reactive oxygen species (ROS). Neutrophils are also able to synthesise proteins, such as cytokines and enzymes, which are stimulated by inflammatory mediators, thus contributing to the inflammatory process (Paape et al., 2003). Functional suppression of neutrophils has been suggested to contribute to periparturient immunosuppression in bovines. The respiratory burst of activity of bovine neutrophils was reduced 1 week prior to parturition, reaching a minimum during the first 2 weeks after calving and returning to normal levels 6 weeks post-calving (Hoeben et al., 2000). Several factors have been proposed to affect neutrophil function, including endocrine and metabolic changes, which occur around calving. An effect of glucocorticoids on neutrophil dysfunction has been suggested; however, it was also demonstrated that corticoids increase the survival of neutrophils *in vitro* (Burton et al., 2005) and enhance the expression of bactericidal gene (Weber et al., 2006). In addition, decreased levels of the glucocorticoid receptor in neutrophils of periparturient cows were observed (Preisler et al., 2000); thus, other factors may contribute to neutrophil dysfunction. Moreover, *in vitro*, 17β-estradiol reduced bovine neutrophil viability only after its migration on epithelial cells, but progesterone or dexamethasone did not show such an effect (Lamote et al., 2006). However, high NEFA concentrations have been shown to increase phagocytosis-associated oxidative bursts in bovine polymorphonuclear (PMN) *in vitro* (Scalia et al., 2006). Similarly, the long chain fatty acids oleic and linoleic increase bovine neutrophil responses, such as granules release, integrin expression and superoxide production in bovine neutrophils (Hidalgo et al., 2011; Mena et al., 2013). Taken together, these antecedents demonstrate that neutrophil activity may be affected by endocrine or metabolic compounds *in vitro*, which are modified during the transition period in cows. However, limited antecedents about the function of neutrophils from transition cows are available. In this study, we isolated neutrophils from transition heifers and assessed the phagocytic and chemotactic activity, lactoferrin release and COX-2 gene expression.

2. Materials and methods

2.1. Animals

Eight healthy primiparous related (half-sisters) Holstein heifers around calving stage (30 days before to 30 after calving), of an age 25 mo, inseminated on the same day with frozen semen from the same bull, body condition scoring 3–3.5, and coming from the same dairy herd of the "Estación Experimental Agropecuaria Austral" farm of the Universidad Austral de Chile, were used. The animals were maintained on an *ad libitum* grass diet supplemented with grain. All experiments were performed according to the protocols approved by the Ethical Committee of the Universidad Austral de Chile.

2.2. Samples and neutrophil isolation

Blood samples were obtained on days 30 and 15 prior to the expectation of calving, at calving and 7, 15 and 30 day post-calving. The samples were obtained in the morning (post-milking after calving) *via* jugular venopuncture into ACD tubes to obtain plasma, isolate neutrophils or into heparin-containing tubes for phagocytosis experiments. The plasma of each sample was immediately stored at −80 °C to measure the NEFA levels. Neutrophils were isolated using the hypotonic lysis method as previously described (Hidalgo et al., 2004). Briefly, the blood was gently mixed for 5 min and then centrifuged at 1000 × *g* at 20 °C for 20 min. The plasma and buffy coat were aspirated and the remaining red blood cell and PMN pellet were suspended in Hank's balanced salt solution (HBSS). The red blood cells were removed by flash hypotonic lysis with a cold phosphate-buffered water solution (0.0132 M, pH 7.2). Upon return to isotonicity with hypertonic phosphate buffer solution (0.0132 M, pH 7.2; 2.7% NaCl), the sample was centrifuged at 600 × *g* at 20 °C for 10 min. The remaining PMN pellet was then washed with HBSS a total of three times. Viability was determined using trypan blue exclusion and was always at least 95%. The neutrophil purity was at least 94%, as assessed using flow cytometry (FACSCanto II, BD, USA) and a forward scatter *versus* side-scatter dot plot to determine the relative size and granularity of the cells (Quinn et al., 2007).

2.3. NEFA analysis

The total NEFA in the plasma samples was measured using a colorimetric method (kit HR Series NEFA-HR, WAKO).

2.4. Phagocytosis assay

Phagocytosis assays were performed using 100 μl of whole blood sample and the pHrodo™ Red *Escherichia coli* BioParticles® Phagocytosis Kit for Flow Cytometry (Life Technology, OR, USA) according to the instructions of the manufacturer. Briefly, 100 μl of blood samples were incubated with 20 μl pHrodo™ BioParticles® Conjugate either on ice (which corresponds to Basal) or at 37 °C (which corresponds to Phagocytosis), for 15 min. After incubation, all tubes were placed on ice, 100 μl of Lysis Buffer A was added and each tube was briefly shaken and incubated at room temperature for 5 min. Then, 1 ml of Buffer B was added, and the tubes were incubated at room temperature for 5 min. The samples were centrifuged at 350 × *g* for 5 min, the supernatants discarded and the cell pellets were resuspended with 1 ml Wash Buffer. The cells were washed one more time with Wash Buffer and finally resuspended in 500 μl of Wash Buffer for flow cytometry analysis. The analysis was performed using a FACSCanto II cytometer (BD Biosciences) with a 488 argon-ion laser. The mean