J. Dairy Sci. 94:2285–2301
doi:10.3168/jds.2010-3371
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Effects of differential supplementation of fatty acids during the peripartum and breeding periods of Holstein cows: II. Neutrophil fatty acids and function, and acute phase proteins

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ABSTRACT

The objectives were to evaluate the effects of differential supplementation of Ca salts (CS) of fatty acids (FA) on plasma acute phase proteins and both FA composition and function (i.e., activity and cytokine production) of neutrophils, during the peripartum and breeding periods. Holstein cows were assigned randomly to receive either CS of palm (PO) or safflower (SO) oils from 30 d prepartum until 35 d postpartum (dpp) and CS of PO or fish oil (FO) from 35 to 160 dpp. Supplementation of CS of FA was at 1.5% of dietary dry matter. Cows (n = 32) were sampled three times weekly from parturition to 35 dpp for analyses of plasma concentrations of haptoglobin and fibrinogen. Cows (n = 47) were sampled for neutrophil phagocytic and oxidative burst activities toward *Escherichia coli* and *Staphylococcus aureus*, and neutrophil abundances of L-selectin and β_2-integrin assessed by flow cytometry at 32 d prepartum, within 7 h after parturition, and 4 and 7 dpp. Profiles of FA in neutrophils and cytokine production (i.e., tumor necrosis factor alpha, TNF-α, and IL-1β) were assessed prepartum (n = 14), 35 (PO vs. SO; n = 26) and 85 dpp (PO vs. FO; n = 28). Plasma concentrations of haptoglobin and fibrinogen were greater for cows fed SO compared with PO. The percentage of neutrophils with phagocytic and oxidative burst activities was not affected by transition diets, but activities per neutrophil were greater in SO compared with PO diets at 4 (phagocytosis and oxidative burst) and 7 dpp (oxidative burst). Neutrophil abundance of L-selectin, but not β_2-integrin, was greater in SO compared with PO at 4 and 7 dpp. Neutrophil productions of TNF-α and IL-1β were increased at 35 dpp in SO compared with PO diets, but production of TNF-α was attenuated in FO compared with PO at 85 dpp. Neutrophil ratios of n-6:n-3 FA were greater at 35 dpp in the SO diet and less at 85 dpp in FO compared with PO diets. In conclusion, cows supplemented with CS of SO had improved innate immunity (i.e., acute phase response and neutrophil function) to better cope with the bacterial challenges in the postpartum period. Conversely, CS of FO attenuated neutrophil cytokine production.

Key words: dairy cow, fatty acid, neutrophil, acute phase protein

INTRODUCTION

Neutrophils are part of the innate immune system acting upon antigens in a nonspecific manner as the first line of defense against pathogens. Migration of neutrophils from the vasculature involves rolling of the neutrophils with adhesion mediated by selectins. After this initial phase, the neutrophil must be activated by chemoattractants for firm adhesion of its integrins to the vascular endothelium. Furthermore, neutrophils internalize and kill microbes by the formation of a phagosome into which reactive oxygen species (**ROS**; O_2^- and H_2O_2) and hydrolytic enzymes are secreted. The consumption of oxygen and generation of ROS is termed the oxidative burst.

Concurrently, the release of inflammatory cytokines, such as tumor necrosis factor α (**TNF-α**) and IL-1β, attract neutrophils to the site of infection, locally stimulate neutrophil phagocytic activity, and systemically stimulate the release of acute phase proteins from the liver (Petersen et al., 2004).

The transition period, typically considered from the time 3 wk prepartum until 3 wk postpartum, is marked by decreasing DMI, negative energy status once lactation is initiated (Staples et al., 1990), and inadequate innate immunity (Kimura et al., 1999; Weber et al., 2001; Kimura et al., 2002; Hammon et al., 2006) that increases the risk of uterine diseases (Kimura et al., 2002; Hammon et al., 2006). Innate immune suppres-

Received April 23, 2010.
Accepted January 21, 2011.
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sion during the transition period is characterized by decreases in both chemotaxis (Kimura et al., 2002) and expression of adhesion molecules (i.e., L-selectin) in neutrophils (Kimura et al., 1999; Weber et al., 2001), and decreased generation of ROS in neutrophils (Kimura et al., 2002; Hammon et al., 2006).

Prostaglandins are derived from the membrane phospholipid stores of arachidonic acid (**C20:4n-6**), which is synthesized from dietary linoleic acid (**C18:2n-6**), an essential fatty acid (**FA**). In the first 10 d postpartum (**dpp**), intense secretion of uterine $PGF_{2\alpha}$ occurs, evidenced by concentrations of plasma 13,14-dihydro-15-keto $PGF_{2\alpha}$ metabolite (**PGFM**; Guilbault et al., 1984), which was elevated in cows that did not develop metritis (Seals et al., 2002; Silvestre et al., 2009). Uterine $PGF_{2\alpha}$ promotes neutrophil chemotaxis and phagocytosis (Hoedemaker et al., 1992).

It was hypothesized that feeding calcium salts (**CS**) of linoleic acid (safflower oil) before parturition and during the first 35 dpp would increase the percentages of linoleic and arachidonic acids in FA profiles of neutrophils, increase neutrophil bactericidal activity and cytokine production, and enhance the acute phase response. Furthermore, feeding CS of eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**; fish oil), beginning at 35 dpp, would attenuate neutrophil cytokine responsiveness. Therefore, our objectives were to investigate the effects of CS of FA on FA profile of neutrophils, surface abundance of adhesion molecules, phagocytic and oxidative burst activities, cytokine production and the systemic acute phase response during the postpartum period. Also, the effects of feeding CS of an n-3 FA-rich supplement on neutrophil cytokine production were examined at approximately 85 dpp.

MATERIALS AND METHODS

Animals, Experimental Design, and Feeding

The present study was part of a large field trial reported in a companion article (Silvestre et al., 2011) that was conducted at a north Florida dairy farm comprising 3,500 Holstein cows milked three times daily. Therefore, information regarding animals, facilities, chemical composition and FA content of diets and supplemental fats are described elsewhere (Silvestre et al., 2011).

Briefly, cows were allocated randomly to the 2 experimental transition diets begun at approximately 30 d before the expected date of parturition and continued until 35 dpp. After 35 dpp, cows within each transition diet were allocated randomly to the 2 experimental breeding diets that were fed until 160 dpp. Experimental transition and breeding diets differed only in the source of supplemental FA.

Cows were evaluated once between 8 to 10 dpp for cervical discharge using a disposable foil-lined cardboard vaginal speculum (Milburn Distributors, Ocala, FL). Appearance of discharge was categorized into clear mucus without flecks, clear mucus with flecks, mucopurulent (50% clear mucus and approximately 50% of pus) and purulent (>50% pus with a brown and foul smell). Additionally, cows were evaluated for BCS on the day of enrollment, parturition, 43 dpp, and at the first insemination (i.e., approximately 87 dpp). Scores were given by 2 veterinarians based on a 1 (thin) to 5 (obese) scale using a quarter scale system (Edmonson et al., 1989).

Transition diets (i.e., prepartum to 35 dpp) consisted of CS of palm oil (**PO**; EnerGII) or CS of safflower oil (**SO**; Prequel 21) and breeding diets consisted of CS of PO (EnerGII) or CS of fish oil (**FO**, StrataG). All CS of FA were manufactured by Virtus Nutrition (Corcoran, CA) and supplemented at 1.5% of the dietary DM. The effects of breeding diets (i.e., FO vs. PO) were tested only in cows supplemented with PO in the transition period. Diets were formulated to meet or exceed the NRC (2001) nutrient requirements for NE_L, CP, fiber, minerals, and vitamins and fed to obtain intakes of 200 and 400 g/d of CS of FA, for pre- and postpartum cows, respectively. Diets were fed as a TMR twice daily targeting 5% orts.

Acute Phase Proteins

Cows [PO (n = 15) and SO (n = 17)] were sampled three times weekly from parturition to 35 dpp for analyses of plasma concentrations of haptoglobin and fibrinogen. Blood samples were collected by puncture of coccygeal vessels into evacuated tubes containing K_2-EDTA (Vacutainer, BD, Franklin Lakes, NJ). Samples were placed immediately into an ice bath until centrifugation for 20 min at 2,619 × *g*. After centrifugation, plasma was harvested and stored frozen at −20°C until assayed.

Plasma haptoglobin concentrations were determined in duplicated samples by measuring haptoglobin/hemoglobin complexing by the estimation of differences in peroxidase activity, as described previously (Makimura and Suzuki, 1982) and used to examine the acute-phase reaction to an endotoxin challenge in beef calves (Carroll et al., 2009). Results are expressed as arbitrary units resulting from the absorption reading at 450 nm. Reference samples were analyzed in duplicate with inter- and intraassay coefficients of variation of 9.0% and 18.0%, respectively.

Plasma fibrinogen concentrations were determined in duplicated samples using a fibrinogen determination kit (Sigma procedure No. 880; Sigma Diagnostics, St. Louis, MO) by estimating clotting time using a BBL Fibrometer coagulation analyzer (BD Diagnostic Systems; Becton, Dickinson and Company, Franklin Lakes, NJ). Results are expressed as mg/dL determined from a standard curve generated from a human fibrinogen reference (Sigma Diagnostics). The intraassay coefficient of variation of the duplicate samples was 2.2%, and the sensitivity of the assay was 86 mg/dL.

Neutrophil Phagocytic and Oxidative Burst Activities

Cows [PO (n = 23) and SO (n = 24)] were sampled at approximately 32 d prepartum, within 7 h after parturition, 4 and 7 dpp for analyses of neutrophil phagocytic and oxidative burst activities by means of a modified dual-color flow cytometry assay (Smits et al., 1997). Bovine whole blood from nonlactating and nonpregnant cows was used for assay optimization of reagents, bacterial concentration, and incubation times. Blood samples were collected by puncture of coccygeal vessels into evacuated tubes containing 10 mL of spray-dried sodium heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Samples were maintained at room temperature and transported to the laboratory for analysis within 4 h of collection. Upon arrival in the laboratory, samples were kept in a rotation station to avoid blood clotting. A 25-μL aliquot was removed from each blood sample for determination of total leukocyte concentration in a hemocytometer after hemolysis of erythrocytes (Unopette, Becton Dickinson). A differential count of neutrophils (i.e., percent neutrophils among total leukocytes) was obtained by smearing 10 μL of whole blood onto a glass slide, stained (Protocol Hema 3, Fisher Diagnostics, Middletown, VA) and examined at a magnification of 40× with oil immersion in which the lateral margins of the smear were included in the differential count. The number of neutrophils per volume of whole blood was calculated based upon total leukocytes multiplied by the percent of neutrophils.

A 100-μL aliquot of blood was pipetted into each of four 5-mL polystyrene round-bottom tubes (12 × 75 mm). Ten microliters of a 5-μ*M* dihydrorhodamine 123 (**DHR**, Sigma-Aldrich) solution (100 μL of DHR stock [500 μ*M*] and 900 μL of PBS) was added to all tubes. Tubes were incubated at 37°C for 10 min on a rotation platform to allow the loading of DHR into the neutrophils. After incubation, tube one was used as a negative control. In tube 2 (positive control) 10 μL of phorbol 12-miristate 13-acetate (PMA**,** Sigma-Aldrich) at a concentration of 2 μg/mL was added to induce in neutrophils an oxidative burst. In tubes 3 (*Escherichia coli*) and 4 (*Staphylococcus aureus*), a volume of a bacterial suspension (10^6 bacteria/μL) was added according to the number of neutrophils to achieve a 40:1 bacteria-to-neutrophil ratio. Bacteria isolates were obtained from 2 cows with mastitis and grown in tryptic soy broth for 18 h at 37°C. Bacteria concentrations were determined by colony counts from serial dilutions of the broth culture and were heat killed (18 h at 37°C) and labeled with propidium iodide (Sigma-Aldrich). All tubes were further incubated at 37°C for 30 min on a rotation platform. After incubation, tubes were placed immediately into ice to stop phagocytosis and oxidative burst activities. Tubes were then processed for flow cytometry using reagents for red blood cell lysis (88% formic acid), white blood cell buffer, and cell fixative (1% paraformaldehyde). An automated lysing system (Q-Prep Epics immunology workstation, Coulter Corp., Miami, FL) was used to add reagents, followed by further addition of 500 μL of distilled water for completion of hemolysis and 10 μL of 0.4% trypan blue solution for quenching extracellular oxidized DHR.

Samples were subjected to flow cytometry (FACSort, Becton Dickinson Immunocytometry Systems, San Jose, CA) utilizing a 488-nm argon-ion laser for excitation and 15 mW of power. Density cytograms were generated by linear amplification of the signals in the forward and side scatter channels. Samples were analyzed within 2 h after addition of fixatives. Neutrophils were gated selectively from acquisition of 10,000 cells/sample based on their sizes and complexity in the density cytogram (Jain et al., 1991). Data were processed and further analyzed by computer software (CellQuest, version 3.3, Becton Dickinson Immunocytometry Systems). Parameters analyzed from the fluorescent cytograms included the percentage of neutrophils that phagocytized bacteria and the percentage of neutrophils with a phagocytosis-induced oxidative burst.

Also, histogram analysis for mean fluorescence intensity (**MFI**) of green (oxidized DHR) and red (propidium iodide-labeled bacteria) wavelengths were used as an estimation of the total gated neutrophil mean oxidative burst intensity (indicator of mean intensity of H_2O_2 produced per neutrophil) and mean phagocytic activity (indicator of mean number of bacteria per neutrophil), respectively.

Adhesion Molecules of Neutrophil and Mononuclear Blood Cells

Abundances of adhesion molecules on neutrophil and mononuclear cells surfaces were analyzed using the same cows and collection days used for neutrophil activities described in the previous section [PO (n = 23) and SO (n = 24)] sampled at approximately 32 d

prepartum, within 7 h after parturition, 4 and 7 dpp). Monoclonal mouse antibodies against bovine L-selectin (**CD62L**, clone CC32, IgG1 isotype; Serotec, Raleigh, NC) and canine β_2-integrin (**CD18**, clone CA1.4E9, IgG1 isotype, Serotec), that cross-reacts with bovine CD18, were used. An isotype mouse control antibody (clone MCA 928, IgG1 isotype, Serotec) was used to correct for nonspecific binding of CD62L and CD18.

Blood samples were collected by puncture of coccygeal vessels into evacuated 10-mL spray-dried sodium heparin tubes. Samples were maintained in room temperature and transported to the laboratory for analysis within 4 h of collection. A total of 10^6 neutrophils in a 100-μL suspension are needed for the immunostaining procedure. Therefore, 4 mL of whole blood (approximately 3×10^6 neutrophils/mL) was added drop-wise into 10 mL of a red blood cell lysing solution of NH_4Cl (168.1 m*M* NH_4Cl, 9.98 m*M* $KHCO_3$, and 0.1293 m*M* EDTA in distilled H_2O) and left at room temperature for 10 min. Samples were centrifuged at $500 \times g$ for 15 min, supernatant decanted, and the cell pellet resuspended in 10 mL of the NH_4Cl solution. Samples were left at room temperature for 10 min and centrifuged at $500 \times g$ for 10 min. The supernatant was decanted and cells resuspended in 10 mL of FACS buffer (2% fetal bovine serum and 0.1% sodium azide in PBS) and centrifuged at $500 \times g$ for 10 min. The supernatant was discarded and cells resuspended in 1 mL of FACS buffer at an approximate concentration of 10^6 neutrophils/100 μL. Cells were kept in an ice bath for the antibody staining procedure.

The cell suspension (100 μL) was added into 3 separate 5-mL tubes for immunostaining of each antibody. Antibodies (12 μL) were added to each individual tube (1:10 dilution of CD62L, CD18, and control antibody in FACS buffer) and refrigerated (3°C) for 30 min. Two milliliters of FACS buffer was pipetted into tubes and centrifuged at $500 \times g$ for 5 min, supernatants were decanted, each tube received 5 μL of phycoerythrin-conjugated goat anti-mouse IgG (STAR81PE, Serotec), and then the tubes were refrigerated (3°C) for another 30 min. Cells were washed with FACS buffer (2 mL) and centrifuged at $500 \times g$ for 5 min. Supernatants were decanted and 0.5 mL of the FACS fixative solution (2% of fetal bovine serum and 0.1% of sodium azide in 0.5% formalin) was pipetted into each tube to resuspend the cell pellet.

A flow cytometer (FACSort) was used to acquire and analyze the neutrophil CD62L and CD18 data. Data from 10,000 events per sample were acquired and analyzed using CellQuest software. The neutrophil and mononuclear cell populations were gated out separately based upon their forward and side scatter characteristics on dot plots (Jain et al., 1991). The percent of neutrophils and mononuclear cells positive for CD62L and CD18 were obtained based upon gated cells and used to further calculate the number of positive cells per volume of blood using hemocytometer cell count results. Also, the geometric mean fluorescent intensity of the labeling kit, an indicator of the number of receptors on the surface of cells, was obtained in the histogram for the gated cell populations.

Neutrophil Isolation, Culture, and FA Analysis

Cows were sampled at enrollment (n = 14), 35 dpp [PO (n = 13) and SO (n = 13)] and at approximately 85 dpp [PO (n = 14) and FO (n = 14)] for analyses of neutrophil FA profiles and cytokine production. Blood samples (150 mL) were collected by puncture of coccygeal vessels into evacuated 10-mL spray-dried sodium heparin tubes, and neutrophils were isolated using the procedure described by Sohn et al. (2007). Briefly, blood was centrifuged at $500 \times g$ for 5 min at 4°C. Plasma, buffy coat, and the top 1/3 of the red blood cells were removed. The remaining red blood cells and white blood cells (~10 mL) were suspended drop-wise into a double volume (20 mL) of cold 0.2% NaCl solution and gently mixed for 5 min to induce lysis of red blood cells. Immediately thereafter, a cold 3.7% NaCl solution, equivalent to half of the original volume of red and white blood cells (5 mL) was added to restore isotonicity. The suspension was centrifuged at $500 \times g$ for 15 min at 4°C, supernatant was discarded and cell pellet resuspended with 5 mL of PBS. Another round of lysis was performed and the cell pellet was washed twice with 20 mL of PBS solution for final resuspension in 2 mL of RPMI-1640 (Bio Whittaker, Walkersville, MD). Cell viability (>98%) was examined in a hemocytometer, and neutrophil purity determined on a glass microscopic slide stained (Protocol Hema 3) for differential cell counts (i.e., >85% neutrophils present). Neutrophils were adjusted to a concentration of 5×10^6 cells/mL in RPMI-1640 and added to a 96-well flat-bottom microtiter plate (100 μL/well) in quadruplicate for each cytokine of interest. Duplicate wells were stimulated or not with 20 μL of LPS stock solution (1 mg/mL; *E. coli* 0111:B4 L3024; Sigma Chemical Co., Saint Louis, MO). The volume per well was adjusted to 200 μL with RPMI-1640 and plates incubated at 37°C, in a 5% CO_2 incubator for 18 h. After incubation, plates were centrifuged at $500 \times g$ for 5 min, supernatants collected, and frozen at −20°C for analysis of TNF-α and IL-1β.

When samples yielded an excess of isolated neutrophils ($>10^8$ neutrophils), cells were centrifuged at 500

× *g* for 5 min, supernatant discarded, cells resuspended in 5 mL of PBS, and stored at −80°C to further determine the FA profile (Jenkins, 2010).

TNF-α ELISA

A TNF-α kit (Bovine TNF-α Screening Set, Thermo Scientific Inc., Rockford, IL) was used to measure TNF-α in supernatants collected from incubations of isolated neutrophils at 35 dpp for cows supplemented with CS of PO (n = 13) or SO (n = 13) during the transition period or at 85 dpp for cows supplemented with CS of PO (n = 14) or FO (n = 14) from 35 to 85 dpp. The TNF- α kit contained coating and biotinylated detection antibodies, recombinant standard, streptavidin-horseradish peroxidase (SR-HRP), 3-3′-5-5′tetramethylbenzidine (**TMB**) substrate solution, and a stop solution. Quadruplicated samples of each animal were analyzed in the same plate that was balanced for numbers of animals from PO and SO or PO and FO diets.

Lyophilized TNF-α coating antibody was reconstituted in Dulbecco's PBS (**DPBS**, pH 7.4) at 300 μg/mL and further diluted (1:100) with carbonate/bicarbonate buffer (0.2 *M*). An aliquot of 100 μL was added to a 96-well microtiter plate (Nunc MaxiSorp C, Fisher Diagnostics), sealed and wrapped in aluminum foil for incubation overnight at 4°C.

After incubation, coating antibody was aspirated by vacuum and the plate washed 3 times with 300 μL of wash solution (150 mL of Tween-20 and 300 mL of DBPS, pH 7.4). An aliquot of 300 μL of blocking buffer (5% of BSA in DPBS) was added to each well. Plates were sealed and wrapped in aluminum foil, and incubated for 1 h at room temperature. Blocking buffer was aspirated and plates washed with 200 μL of wash solution. The bovine standard of TNF-α was serially diluted in reagent diluents (4% BSA in DPBS, pH 7.4; 39 to 2,500 pg/mL), and 100 μL of standards and experimental samples were added to respective wells and incubated for 1 h at room temperature. Plates were washed 3 times with 200 μL of wash solution. Reconstituted TNF-α detection antibody (250 μL/mL) was diluted in reagent diluent (1:100) and added to each well for 1-h incubation at room temperature and washed 3 times with 200 μL of wash solution. The SR-HRP reagent was diluted in reagent diluents (1:400), 100 μL added to each well, and incubated for 30 min at room temperature in the dark. Plates were washed 3 times with 200 μL of wash solution, and TMB substrate solution (100 μL) added to each well for 2 min in the dark. The reaction was stopped (100 μL of 2 *M* sulfuric acid) and absorbance read at 450 nm. An internal standard diluted in RPMI-1640 was added to each plate in duplicate to permit inclusion of assay plate in the statistical analysis. The coefficient of variation of duplicate samples in a dose titration analysis for LPS in RPMI-1640 media was 4.3%. The coefficient of variation determined from analysis of duplicate samples of experimental cultures was 2.3%.

IL-1β ELISA

Measurement of IL-1β was performed in a 96-well microtiter plate and using wash solution (Tris-buffered saline, 0.05% Tween-20, pH 8.0) and TMB substrate obtained from Bethyl Laboratories (ELISA Starter Kit, Montgomery, TX). Coating (MCA1658), detection (AHP423) and HRP-conjugated (STAR54) antibodies were obtained from Serotec (Raleigh, NC). Standard recombinant bovine IL-1β was obtained from Thermo Scientific (Rockford, IL). All samples of each animal were analyzed in the same plate that was balanced for numbers of animals for PO and SO or PO and FO diets.

Plates were coated overnight at 4°C with 5 μg/mL of mouse anti-bovine IL-1β in carbonate coating buffer (Bethyl Laboratories), washed 3 times (300 μL), and blocked [100 μL/well, 2% fish skin gelatin (45% solution; Sigma-Aldrich) in wash solution] for 1 h at room temperature. Blocking buffer was aspirated and plates washed. Standards of IL-1β were diluted serially in wash solution (27 to 20,000 pg/mL). Serially diluted standards and experimental neutrophil culture supernatants were added (100 μL) to wells and incubated for 2 h at room temperature. Plates were washed 3 times. Bovine IL-1β detection antibody, diluted in wash solution (1:500), was added (100 μL) to each well for 1-h incubation at room temperature and washed 3 times. Anti-IL-1β HRP-conjugated antibody diluted in wash solution containing 0.2% fish gelatin (1:500) was added to each well (100 μL) and incubated for 30 min at room temperature in the dark. Plates were washed 3 times and TMB substrate solution (100 μL) added to each well for 5 min in the dark. The reaction was stopped (100 μL of 2 *M* sulfuric acid) and absorbance read at 450 nm. An internal standard was added to each plate in duplicate to permit inclusion of assay plate in the statistical analysis. The coefficient of variation determined from analysis of duplicate samples of experimental cultures was 3.3%.

Statistical Analyses

Plasma concentrations of acute phase proteins (i.e., haptoglobin and fibrinogen), neutrophil activity (i.e., percent phagocytosis and oxidative burst, MFI phagocytosis or oxidative burst), and expression of neutrophil adhesion molecules (i.e., CD62L and CD18) were ana-

lyzed using repeated measures responses of the mixed model procedure of SAS (SAS Institute, Inc.; Version 9.1). Data were tested for normal distribution of the residuals by the PROC UNIVARIATE procedure of SAS. Residuals were considered to be normally distributed when the Shapiro-Wilk statistic was equal or greater than 0.10 and log-transformed if not normally distributed. For each dependent variable, the covariance structure that had the best relative goodness of fit based upon penalty criteria (Bayesian criterion) was used. The mathematical model contained diet, dpp, and diet by dpp interaction with cow (random variable) nested within diet. For responses that can be affected by inflammation (i.e., acute phase proteins, neutrophil activity, and adhesion molecule expression) and because vaginoscopy score was a significant variable, vaginoscopy score was retained in the statistical model for estimating potential diet effects. Furthermore, cows with purulent and mucopurulent discharge also were excluded from the data set for additional analyses to avoid confounding.

Analyses of production of cytokines (i.e., TNF-α and IL-1β) by neutrophils were conducted by the method of least squares using the general linear model (GLM) procedure of SAS. Cytokine concentrations were compared within LPS nonstimulated, LPS stimulated, and increased mass (i.e., LPS stimulated minus LPS nonstimulated). The final mathematical model consisted of diet.

RESULTS

Acute Phase Proteins

A total of 15 and 17 multiparous cows fed PO and SO diets, respectively, were used for analysis of acute phase proteins. Ten cows in each transition diet were excluded because sampling was terminated during the postpartum period after antibiotic or anti-inflammatory treatments. Cows in the SO group were fed experimental diets for a longer period (34.8 $\pm$ 1.4 d) compared with the PO group (30.8 $\pm$ 1.3 d) before parturition. Frequency distribution of cows among BCS on the day of enrollment [2.5 to 2.75 (n = 16); 3.0 to 3.25 (n = 14); and 3.75 (n = 2)] and at parturition [2.5 to 2.75 (n = 8); 3.0 to 3.25 (n = 20); and 3.5 to 3.75 (n = 4)] did not differ between PO and SO diets. All cows had a normal parturition (i.e., no assistance), except for 1 cow that had minor assistance. A total of 10, 2, 1, and 2 cows fed the PO and 9, 5, 0, and 3 cows fed the SO diets were diagnosed with either clear mucus without flecks or lochia, clear mucus with flecks, mucopurulent or purulent cervical discharges at 8 dpp, respectively, and were not treated with antibiotics or anti-inflammatory agents.

Figure 1. Least squares means (±SEM) for plasma concentrations of haptoglobin (A) and fibrinogen (B), adjusted for vaginoscopy score with inclusion in the mixed model analyses, for cows supplemented with calcium salts of palm oil (■; n = 15) or safflower oil (▲; n = 17) during the prepartum period (at least 20 d) to 35 d postpartum (dpp). $^*P < 0.01$.

Mean plasma concentrations of haptoglobin and fibrinogen were greater ($P < 0.05$) for cows fed SO compared with PO transition diets (Figure 1, A and B). Cows diagnosed with cervical discharge classified as clear mucus with or without flecks or lochia had lesser ($P < 0.05$) mean concentrations of haptoglobin and fibrinogen compared with cows diagnosed with mucopurulent or purulent discharges (Figure 2, A and B).

An additional analysis was conducted only with cows that had cervical discharges as clear mucus with or without flecks or lochia (n = 26). Plasma concentrations of haptoglobin and fibrinogen were greater ($P <$ 0.01) for cows fed SO (0.02 $\pm$ 0.002 AU, 2.217 $\pm$ 0.11 mg/mL) compared with PO (0.01 $\pm$ 0.002 AU, 1.725 $\pm$ 0.12 mg/mL) transition diets, respectively.

oil that is rich in linoleic acid (Tappia et al., 1997). Additionally, the greater concentration of fibrinogen in the blood samples of cows supplemented with SO may have benefitted the phagocytic and oxidative burst activity of the neutrophil because whole blood was used without the need of cell isolation. Indeed, in vitro studies (Shi et al., 2001; Rubel et al., 2002) have shown that fibrinogen can profoundly alter leukocyte function, leading to changes in cell migration, phagocytosis, NFκB-mediated transcription, production of chemokines and cytokines, and degranulation.

Changes in the FA profile of the neutrophils, favoring a greater ratio of n-6:n-3 FA could have led to a greater production of inflammatory mediators, such as prostaglandins. Lymphocyte stimulation with concavalin A, a mitogen, resulted in a decrease in the linoleic acid fraction, possibly reflecting its utilization as a precursor of eicosanoids. Eicosanoids stimulate NFκB for nuclear translocation (Camandola et al., 1996), resulting in increased TNF-α and IL-1β production by monocytes (Baldie et al., 1993, Sinha et al., 1991). Indeed, neutrophil activity increased positively with increasing numbers in the blood. More neutrophils may result in a greater production of eicosanoids and cytokines in the confines of the in vitro system. The greater production of these mediators can stimulate in an autocrine manner the activity of neutrophils. Moreover, the lesser number of neutrophils per volume of blood in the SO diet was sufficient to not affect percent neutrophil activity, although number of bacteria engulfed per neutrophil was increased in this group of cows.

Suppression of TNF-α and a numerical decrease of IL-1β production in cows supplemented with FO was possibly due to an increased proportion of the n-3 family of FA (i.e., EPA and DHA), with a concurrent decrease in n-6 FA within the neutrophils. The n-3 FA, especially EPA and DHA, can compete for intracellular enzymatic pathways to generate prostaglandins of the 3 series and leukotriene B_5, with both classes having anti-inflammatory properties (Mattos et al., 2000). Indeed, EPA and DHA inhibited production of IL-1β and of TNF-α by human monocytes (Sinha et al., 1991; Purasiri et al., 1997). Also, Caughey et al. (1996) demonstrated that a diet enriched with flaxseed, followed by FO, inhibited IL-1 and TNF-α production by monocytes, which was negatively correlated with the EPA content in the FA profile of these cells.

Supplementation of fish meal, an enriched source of the n-3 FA, improved pregnancy per AI in a few studies (Armstrong et al., 1990; Carroll et al., 1994; Burke et al., 1997). Also, Petit and Twagiramungu (2006) observed that embryonic mortality from d 30 to 50 post insemination tended to be decreased in cows that received linseed compared with cows that received CS of PO, indicative that linseed improved embryonic survival after embryonic attachment to the uterus, which occurs gradually, beginning at approximately 17 to 25 d of pregnancy.

In addition to the possible suppression of omega-3 FA on $PGF_{2\alpha}$ secretion (Mattos et al., 2003; Mattos et al., 2004) and possible attenuation of luteolysis during the time of pregnancy recognition, greater pregnancy rates and lesser pregnancy losses in cows supplemented with FO were observed (Silvestre et al., 2011). The possibility of progesterone being a confounding factor for the immune suppression of neutrophils was excluded, because all animals were sampled 2 to 3 d after the last $PGF_{2\alpha}$ injection of the Ovsynch protocol. An immunological suppression or tolerance is needed during pregnancy; the allograft embryo/fetus-placental unit must avoid the mother's rejection for the duration of pregnancy (Siiteri and Stites, 1982). An immune response to a foreign body starts with the induction of an inflammatory response that is amplified by cytokines produced by cells (i.e., epithelial cells, macrophages and later neutrophils) in the vicinity of the foreign body.

In conclusion, CS of SO, a fat supplement rich in linoleic acid, can decrease the threshold for triggering an immune response (i.e., creating a pro-inflammatory state that can respond greatly upon challenge) that alters innate immunity (i.e., acute phase response and neutrophil function). This pro-inflammatory state might be suitable for coping with the stressful and highly contaminated postpartum period. Conversely, CS of FO can increase the threshold for triggering an immune response during the breeding period, exerting an anti-inflammatory environment that may attenuate immune responses in early pregnancy upon environmental challenges (i.e., mastitis, heat stress) that may benefit embryonic survival.

ACKNOWLEDGMENTS

The authors express their appreciation to Ron St.-John, Peter Gelber, and the staff at Alliance Dairies (Trenton, FL) for expert management of experimental cows. This research was supported partially by grants from Virtus Nutrition Inc. and the National Research Initiative USDA Research Grant #2004-35203-14137.

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