Ethyl-cellulose rumen-protected methionine alleviates inflammation and oxidative stress and improves neutrophil function during the periparturient period and early lactation in Holstein dairy cows

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ABSTRACT

The periparturient period is the most critical phase in the productive cycle of dairy cows and is characterized by impairment of the immune system. Our objective was to evaluate the effect of feeding ethyl-cellulose rumen-protected methionine (RPM) starting at d –28 from expected parturition through 60 d in milk on biomarkers of inflammation, oxidative stress, and liver function as well as leukocyte function. Sixty multiparous Holstein cows were used in a block design and assigned to either a control or the control plus ethyl-cellulose RPM (Mepron, Evonik Nutrition & Care GmbH). Mepron was supplied from –28 to 60 d in milk at a rate of 0.09% and 0.10% dry matter during the prepartum and postpartum period. That rate ensured that the ratio of Lys to Met in the metabolizable protein was close to 2.8:1. Blood samples from 15 clinically healthy cows per treatment were collected at d –30, –14, 1, 7, 21, 30, and 60 and analyzed for biomarkers of liver function, inflammation, and oxidative stress. Neutrophil and monocyte function in whole blood was measured in vitro at –14, 1, 7, 21, and 30 d in milk. The statistical model included the random effect of block and fixed effect of treatment, time, and its interaction. Compared with control, ethyl-cellulose RPM increased plasma cholesterol and paraoxonase after parturition. Among the inflammation biomarkers measured, ethyl-cellulose RPM led to greater albumin (negative acute-phase protein) and lower haptoglobin than control cows. Although concentration of IL-1 β was not affected by treatments, greater IL-6 concentration was detected in response to ethyl-cellulose RPM. Cows supplemented with ethyl-cellulose RPM had greater

plasma concentration of ferric-reducing antioxidant power, β -carotene, tocopherol, and total and reduced glutathione, whereas reactive oxygen metabolites were lower compared with control cows. Compared with control, ethyl-cellulose RPM enhanced neutrophil phagocytosis and oxidative burst. Overall, the results indicate that ethyl-cellulose RPM supply to obtain a Lys-to-Met ratio of 2.8:1 in the metabolizable protein during the periparturient period and early lactation is an effective approach to help mitigate oxidative stress and inflammation as well as enhance liver and neutrophil function in dairy cows.

Key words: immunometabolism, methionine, transition period

INTRODUCTION

The periparturient period is the most critical phase in the productive life of high-producing dairy cows, and is characterized by the highest incidence and severity of both metabolic and infectious diseases (Bertoni et al., 2008; Trevisi et al., 2012; Sordillo, 2016). The inflammatory response can be activated by infection or injury, as well as tissue stress and malfunction in the absence of infection or evident tissue damage (Chovatiya and Medzhitov, 2014; Trevisi et al., 2016). The classic initiators of inflammation, infection and tissue injury, trigger the recruitment of leukocytes to the damaged or contaminated tissue, and immune cell function is fundamental for the efficiency and duration of the inflammatory process (Medzhitov, 2008). In cases of tissue stress or malfunction, the inflammatory response is mainly dependent on tissue-resident macrophages and, based on human disease models, it is responsible for chronic inflammatory conditions (Medzhitov, 2008). The inflammatory process triggers the acute-phase response, which alters liver function at least in part by switching to synthesis of positive acute-phase proteins

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(e.g., ceruloplasmin, haptoglobin) instead of negative acute-phase proteins (e.g., albumin) in an attempt to reestablish homeostasis (Cray et al., 2009).

Most dairy cows in early lactation experience a prolonged imbalance between production of free radicals and their elimination by antioxidants (Sordillo and Aitken, 2009), often causing cellular damage followed by a chronic inflammatory response. Oxidative stress around calving also can be a consequence of an infection-induced inflammatory response (Trevisi et al., 2016). Thus, the inflammatory events that afflict cows during early lactation are associated with physiological stressful conditions and the metabolic overload of the liver to meet the nutrient requirements for maintenance and milk synthesis (Trevisi et al., 2016).

It is well established that transition cows experience negative energy and protein balance (Drackley, 1999), and that nutrition plays a critical role in supporting the immune system (Bertoni et al., 2015; Sordillo, 2016). Methionine is the first-limiting AA in dairy cows (NRC, 2001), and recent data underscored its immunometabolic potential (Osorio et al., 2014; Sun et al., 2016; Zhou et al., 2016a). In the liver, methionine can be metabolized to S-adenosyl methionine, a fundamental methyl donor, as well as phosphatidylcholine, which is a main constituent of very low density lipoproteins. Furthermore, methionine metabolism can generate intracellular antioxidants such as glutathione (**GSH**) and taurine (Brosnan and Brosnan, 2006). Therefore, our general hypothesis was that feeding ethyl-cellulose rumen-protected methionine (**RPM**) to obtain a Lys-to-Met ratio of 2.8:1 in the MP from d -28 relative to the expected parturition to 60 DIM would help reduce the inflammatory and oxidative stress status and enhance liver function and leukocyte function in the cow.

MATERIALS AND METHODS

Animal Housing and Care

The Institutional Animal Care and Use Committee at the University of Illinois (Urbana; protocol #14270) approved all experimental procedures. All cows were housed in a freestall system equipped with the Calan gate system (American Calan Inc., Northwood, NH) during the prepartum period. After parturition, all cows were housed in tiestalls. Cows were fed once daily (1300 h) at 120% of expected intake, and milked 3 times daily (0600, 1400, and 2200 h).

Design and Treatments

Additional details of the experimental design have been described previously (Batistel et al., 2017). Briefly,

60 multiparous Holstein cows from the University of Illinois Dairy Research Farm were used in a randomized, complete, unbalanced block design experiment with 30 cows per treatment. The data presented herein is from a subset of cows. Cows were blocked by the expected parturition day, and the blocks were balanced by parity, previous 305-d milk yield, and BCS. The BCS used to block cows was measured at -30 d before parturition using the scale 1 to 5 (1 = thin, 5 = fat; scale in 0.25 increments). Cows within each block were randomly assigned to 1 of the 2 treatments. Treatment diets were a control diet without Met supplementation or the control diet plus ethyl-cellulose RPM (Mepron, Evonik Nutrition & Care GmbH, Hanau-Wolfgang, Germany). Ethyl-cellulose RPM was supplied from -28 to 60 d relative to parturition at a rate of 0.09 and 0.10% of the DMI of the previous day during the prepartum and postpartum period. Mepron is a commercial rumen-protected source of DL-Met that resists ruminal degradation through an ethyl-cellulose film coating. Pellets measure 1.8×3 mm and contain 85% DL-Met. The intestinal digestibility coefficient and rumen bypass of Mepron are 90 (Schwab, 1995) and 80% (Overton et al., 1996), respectively; therefore, per 10 g of Mepron, the cows received 6.1 g of Met available for absorption. The target values of Met supply were based on recent experiments demonstrating a benefit in terms of production performance and health of supplementing rumen-protected Met to achieve a Lys-to-Met ratio close to 2.8:1 during the prepartum and postpartum periods (Osorio et al., 2013; Zhou et al., 2016b). The Lys-to-Met ratio in the control group was close to 3.7:1 during the prepartum and postpartum periods. During the far-off period (from -45 to -29 d), all cows received the same diet (1.33 Mcal/kg of DM and 13.9% CP) with no RPM. The basal close-up (from -28 d to parturition), fresh (from 1 to 30 d), and high-production (from 31 to 60 d) diets contained 1.47 Mcal/kg of DM and 15.3% CP, 1.67 Mcal/kg of DM and 17.7% CP, and 1.61 Mcal/kg of DM and 17.4% CP, respectively. The ingredient and nutrient composition of the diets fed are reported in Table 1 and 2. All diets were formulated to meet cow predicted requirements according to NRC (2001).

Blood and Liver Sample Collection and Biomarker Analysis

Blood was sampled from the coccygeal vessel before the feeding in 15 cows per treatment at d -30, -14, 1, 7, 21, 30, and 60 d relative to calving date. This subset of cows was chosen based on absence of clinical disease and enrollment for liver biopsy. Incidence of ketosis, lameness, mastitis, milk fever, retained placenta, and displaced abomasum did not differ with treatment (Ba-

tistel et al., 2017). Liver biopsy cows were chosen based on previous 305-d milk yield, BCS, and absence of clinical disease. Samples were collected into evacuated tubes (BD Vacutainer, BD and Co., Franklin Lakes, NJ) containing lithium heparin. After blood collection, samples were placed on ice until centrifugation (~40 min) or the neutrophil function assay. Plasma was obtained by centrifugation at $2,000 \times g$ for 30 min at 4°C. Aliquots of plasma were frozen (−80°C) until further analysis.

Liver was sampled via puncture biopsy (Dann et al., 2005) from 10 cows per treatment. Samples were collected from the same cows under local anesthesia at approximately 0800 h on d −21, −10, 1, and 10 relative

to parturition. Liver was frozen immediately in liquid N and stored (−80°C) until further analysis.

Plasma samples were analyzed for albumin (catalog no. 0018250040), aspartate aminotransferase (catalog no. 0018257540), bilirubin (catalog no. 0018254640), and cholesterol (catalog no. 0018250540) with kits purchased from Instrumentation Laboratory Spa (Werfen Co., Milan, Italy) using the clinical auto-analyzer (ILAB 650, Instrumentation Laboratory). Haptoglobin, ceruloplasmin, and paraoxonase were determined by adapting the methods of Owen et al. (1960) and Skinner et al. (1991), Sunderman and Nomoto (1970), and Ferré et al. (2002), respectively, to the ILAB 650 conditions. Reactive oxygen metabolites (**ROM**) were ana-

Table 1. Ingredient and nutrient composition of far-off (from −45 to −29 d), close-up (from −28 d to parturition), fresh (from 1 to 30 DIM) and high-producing (from 31 to 60 DIM) diets

Ingredient, % of DM	Diet			
	Far-off	Close-up	Fresh	High-producing
Alfalfa haylage	—	6.55	7.81	10.8
Corn silage	34.7	26.6	31.0	31.9
Wheat straw	33.7	26.5	3.25	—
Corn grain, ground, dry	—	12.6	22.21	20.7
Cottonseed	—	—	2.17	1.83
Molasses, beet sugar	—	4.03	5.50	4.51
Soybean hulls	15.7	3.46	4.25	9.96
Soybean meal, 48% CP	12.0	7.83	10.1	7.98
Expeller soybean meal ¹	—	5.80	5.16	5.17
Protein supplement ²	—	0.78	1.81	1.58
Urea	0.46	0.59	0.39	0.40
Soychlor ³	—	1.23	—	—
Saturated fat supplement ⁴	—	—	2.25	2.14
Limestone	—	—	1.41	0.96
Salt	0.40	—	0.02	0.04
Dicalcium phosphate	0.50	0.52	1.17	0.92
Magnesium oxide	—	—	0.08	0.04
Magnesium sulfate	1.90	2.08	0.02	—
Sodium bicarbonate	—	—	0.84	0.59
Mineral vitamin mix ⁵	0.40	0.17	0.17	0.20
Vitamin A ⁶	—	0.03	0.02	0.02
Vitamin D ⁷	—	0.03	—	—
Vitamin E ⁸	0.40	0.60	—	—
Biotin ⁹	—	0.70	0.42	0.32
Monensin ¹⁰	0.01	—	—	—
Ethyl-cellulose RPM ¹¹	—	0.09	0.10	0.10

¹SoyPlus, West Central Soy, Ralston, IA.

²ProVAAl AADvantage, Perdue AgriBusiness, Salisbury, MD.

³West Central Soy.

⁴Energy Booster 100, Milk Specialties Global, Eden Prairie, MN.

⁵Contained a minimum of 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5,000 mg of Cu/kg, 250 mg of I/kg, 40 mg of Co/kg, 150 mg of Se/kg, 2,200 kIU of vitamin A/kg, 660 kIU of vitamin D₃/kg, and 7,700 IU of vitamin E/kg.

⁶Contained 30,000 kIU/kg.

⁷Contained 5,000 kIU/kg.

⁸Contained 44,000 kIU/kg.

⁹ADM Animal Nutrition, Quincy, IL.

¹⁰Rumensin, Elanco Animal Health, Greenfield, IN.

¹¹Ethyl-cellulose rumen-protected methionine, Evonik Nutrition & Care GmbH, Hanau-Wolfgang, Germany. Only added in the treatment group.

Table 2. Nutrient composition and diet evaluation using NRC (2001) for prepartal and postpartal diets fed to multiparous Holstein cows supplemented with ethyl-cellulose rumen-protected methionine (RPM)

Item	Close up		Fresh		High production	
	Far-off	Control	RPM	Control	RPM	RPM
Chemical composition, ¹ % DM						
CP	13.9 ± 0.25	15.6 ± 0.32	15.7 ± 0.32	17.7 ± 0.36	17.4 ± 0.36	17.6 ± 0.36
NDF	54.5 ± 0.75	40.8 ± 0.68	40.7 ± 0.68	29.2 ± 0.59	29.3 ± 0.59	31.4 ± 0.62
ADF	36.9 ± 0.65	27.5 ± 0.50	27.4 ± 0.50	19.5 ± 0.38	19.6 ± 0.38	21.4 ± 0.48
NFC	24.7 ± 0.72	34.9 ± 0.81	34.9 ± 0.81	41.4 ± 0.55	40.7 ± 0.54	40.9 ± 0.54
Ether extract	1.81 ± 0.04	2.32 ± 0.05	2.33 ± 0.05	5.12 ± 0.14	5.13 ± 0.14	5.15 ± 0.14
Calculated with NRC (2001) ²						
NE _L , Mcal/kg of DM	1.33	1.47	1.47	1.67	1.6	1.6
NE _L allowable milk, kg/d	—	—	—	32.3	42.8	45.3
MP allowable milk, kg/d	—	—	—	38.8	49.4	51.7
RDP, % of DM	8.8	9.4	9.4	10.2	9.9	10
RUP, % of DM	5.1	6.2	6.3	7.4	7.4	7.6
RDP required, g/d	1,149	1,194	1,196	2,003	2,505	2,512
RDP supplied, g/d	1,157	1,204	1,203	2,014	2,508	2,518
RDP balance, g/d	8	10	7	11	3	7
RUP required, g/d	131	119	130	1,599	1,904	2,150
RUP supplied, g/d	668	793	924	1,461	1,876	2,022
RUP balance, g/d	537	673	794	-138	-28	-128
MP required, g/d	790	808	808	2,543	3,078	3,200
MP supplied, g/d	1,211	1,363	1,473	2,425	3,055	3,134
MP balance, g/d	421	555	664	-118	-23	-66
Lys, % of MP	6.74	6.54	6.51	6.40	6.33	6.29
MP-Lys, g	82	89	89	155	194	195
Met, % of MP	1.77	1.73	2.30	1.70	1.68	2.22
MP-Met, g	21	24	32	41	52	68
Lys:Met	3.81:1	3.71:1	2.81:1	3.78:1	3.78:1	2.82:1

¹Mean ± SD.²The NRC (2001) evaluation of diets was based on final averaged pre- and postpartum DMI, production data, and feed analysis.

lyzed with the d-ROMs-test (cod. MC002) purchased from Diacron (Grosseto, Italy). Total antioxidant capacity was assessed as ferric-reducing antioxidant power (**FRAP**) using the colorimetric method of Benzie and Strain (1996). Myeloperoxidase was determined as previously-described (Bionaz et al., 2007), via colorimetry based on the reaction of MPO contained in the plasma sample with hydrogen peroxide, which forms H_2O and O^- ; the *o*-dianisidine dihydrochloride, and electron donor, reacts with the O^- , releasing H_2O and a colored compound. Plasma retinol and tocopherol were extracted with hexane and analyzed by reverse-phase HPLC using Spherisorb ODS-2, 3 μm , in a 150×4.6 mm column (Alltech, Deerfield, IL); a UV detector set at 325 (for retinol) or 290 nm (for tocopherol); and 80:20 methanol:tetrahydrofuran as the mobile phase. Bovine IL-1 β (Cat. No. ESS0027; Thermo Scientific, Rockford, IL) and IL-6 (Cat. No. ESS0029; Thermo Scientific) plasma concentrations were determined using commercial kits.

The simultaneous phagocytosis capacity and oxidative burst activity of peripheral monocytes and neutrophils was determined upon challenge with enteropathogenic bacteria (*Escherichia coli* 0118:H8) exactly as described previously (Zhou et al., 2016a). Data are reported as percentages of CD14- and CH138A-positive cells with phagocytosis and oxidative burst capability.

Liver tissue total and oxidized GSH were measured using a commercial kit (Cat. No. NWH-GSH01; Northwest Life Science Specialties LLC, Vancouver, WA). Reduced GSH was calculated as reduced GSH = total GSH – oxidized GSH.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS v.9.4 (SAS Institute Inc., Cary, NC) according to the following model with repeated measures:

$$Y_{ijkl} = \mu + b_i + M_j + T_k + MT_{jk} + c_l(b_i) + e_{ijkl}$$

where Y_{ijkl} = dependent, continuous variable, μ = overall mean, b_i = random effect of block ($i = 1$ to 7), M_j = fixed effect of treatment (j = control vs. ethyl-cellulose RPM), T_k = fixed effect of time (day or week), MT_{jk} = interaction between treatment and time, $c_l(b_i)$ = random effect of cow within block, and e_{ijkl} = residual error. The covariates for parity, previous 305-d milk yield, and BCS at -30 d relative to parturition were kept in the model for all variables when significant. For the blood analysis, plasma data at -30 d relative to parturition were included as a covariate when significant. Unless otherwise specified, first-order autoregressive was the

covariate structure used for analysis because it resulted in the lowest Bayesian information criterion for most of the variables measured. Normality of the residuals was checked with normal probability and box plots. Data were $\log_2$ -scale transformed if needed to comply with normal distribution of residuals. For ease of interpretation, the tables and graphs contain the $\log_2$ back-transformed means that resulted from the statistical analysis. Significance was determined at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS AND DISCUSSION

Biomarkers of Liver Function

During the transition to lactation, the metabolic activity of liver increases substantially in large part to help meet the nutrient requirements necessary for milk synthesis. The glucose requirement has been estimated to be 1.2 kg/d at 3 wk before calving and increases to 3 kg/d as a result of hepatic gluconeogenesis at 3 wk postpartum when milk production is around 36 kg/d (Reynolds et al., 2003; Aschenbach et al., 2010). The liver also is essential for the metabolism of nonesterified fatty acids taken up due to the mobilization of adipose depots (Drackley et al., 2001). Thus, cows with optimal liver function are able to overcome the periparturient period more efficiently and exhibit signs of reduced inflammation, oxidative, and metabolic stress (Bionaz et al., 2007; Bertoni et al., 2008).

During the periparturient period, cows experience several stressful events including regrouping, dietary changes, parturition, and the onset of lactation (Trevisi et al., 2016). Under normal physiological conditions the liver synthesizes and releases proteins and enzymes into the blood, which can be used as biomarkers of liver function (Ferré et al., 2002; Bertoni et al., 2008). Main effects of ethyl-cellulose RPM, time, and interactions on liver function biomarkers are reported in Table 3. Ethyl-cellulose RPM increased plasma paraoxonase ($P < 0.01$) and cholesterol ($P = 0.02$) after parturition (Figure 1), but aspartate aminotransferase and bilirubin were not affected by treatments ($P \geq 0.15$).

Similar to our results, a recent study reported that, although basal plasma paraoxonase concentration decreased soon after parturition, cows supplemented with RPM during the periparturient period had greater liver synthesis of paraoxonase during the postpartum period (Zhou et al., 2016a). Although the higher plasma concentrations of cholesterol in the ethyl-cellulose RPM group reflected in part the greater DMI of those cows, its response together with the higher plasma concentrations of paraoxonase and lower γ -glutamyl transferase

(Batistel et al., 2017) indicate that ethyl-cellulose RPM supplementation was able to ameliorate liver function during the postpartum period.

Biomarkers of Inflammation

Main effects of ethyl-cellulose RPM, time, and interactions on inflammation biomarkers are reported in Table 3. The inflammatory process is a well-conserved mechanism to overcome adverse stimuli and restore homeostasis. Dairy cows experience some degree of inflammation after parturition (Bertoni et al., 2004; Humblet et al., 2006; Sordillo, 2016). A retrospective study, where cows were classified by degree of liver inflammation based on plasma concentrations of acute-phase proteins, indicated that inflammation increases the risk of transition disorders and impairs animal performance during early lactation (Bertoni et al., 2008). In a recent review, Bradford et al. (2015) proposed that the duration of the inflammatory process rather than its complete avoidance is one of the normal adaptations to lactation; therefore, measuring the consequences of inflammatory conditions in the peripartum period is of importance (Trevisi et al., 2016).

Among the inflammation biomarkers measured, compared with control, ethyl-cellulose RPM led to greater

($P < 0.01$) albumin (negative acute-phase protein) and lower haptoglobin ($P < 0.01$; Figure 2). This marked decrease in the concentration of haptoglobin by d 21 postpartum in ethyl-cellulose RPM-supplemented cows indicates they were able to better control the inflammatory response. Additionally, the higher concentration of plasma albumin, as well as cholesterol and paraoxonase, which are also biomarkers of anti-inflammatory responses, in ethyl-cellulose RPM cows supports the idea that they were under a less pronounced inflammatory state (Trevisi et al., 2011; Grossi et al., 2013). Myeloperoxidase tended ($P = 0.15$) to be higher in the ethyl-cellulose RPM group compared with control cows. Ceruloplasmin and alkaline phosphatase were not affected by ethyl-cellulose RPM supply. For cytokines, a main effect with greater IL-6 in ethyl-cellulose RPM-supplemented cows was detected ($P = 0.05$), whereas the concentration of IL-1 β was not affected ($P > 0.10$) by ethyl-cellulose RPM supply. Because IL-6 has pro-inflammatory and anti-inflammatory properties (Xing et al., 1998; Scheller et al., 2011), the increased albumin and decreased haptoglobin profiles suggest that in ethyl-cellulose RPM cows it might have exerted an anti-inflammatory effect. A similar pattern of IL-6 was observed when periparturient cows received a different source of RPM (Zhou et al., 2016a).

Table 3. Effects of ethyl-cellulose rumen-protected methionine (RPM) supplementation to multiparous Holstein dairy cows during the close-up (from -28 d to parturition), fresh (from 1 to 30 DIM), and high-producing (from 31 to 60 DIM) periods on plasma biomarkers of liver function, inflammation and oxidative stress

Item	Control	RPM	SEM	P-value		
				Treatment ¹	Time	Treatment × time
Liver function						
Aspartate aminotransferase, U/L	85.4	95.1	6.01	0.89	<0.01	0.92
Bilirubin, μmol/L	3.74	3.39	0.34	0.48	<0.01	0.80
Cholesterol, mmol/L	3.50	4.04	0.11	0.02	<0.01	0.46
Paraoxonase, U/mL	84.0	94.8	3.54	<0.01	<0.01	0.36
Inflammation						
Alkaline phosphatase, U/L	57.4	56.1	1.53	0.57	<0.01	0.77
Albumin, g/L	36.4	38.1	0.38	<0.01	<0.01	0.48
Ceruloplasmin, μmol/L	2.65	2.67	0.09	0.82	<0.01	0.32
Haptoglobin, g/L	0.55	0.46	0.04	0.05	<0.01	0.27
Myeloperoxidase, U/L	362	380	6.04	0.15	<0.01	0.45
IL-6, pg/mL	672	874	94.9	0.05	0.02	0.46
IL-1β, pg/mL	5.21	4.89	1.15	0.24	<0.01	0.57
Oxidative stress						
FRAP, ² μmol/L	139	144	2.45	0.04	<0.01	0.92
ROM, ³ H ₂ O ₂ /100 mL	16.8	16.1	0.31	0.01	<0.01	0.95
β-Carotene, mg/100 mL	0.17	0.23	0.02	0.02	0.01	0.33
Retinol, μg/mL	27.0	28.4	0.72	0.18	0.19	0.96
Tocopherol, μg/mL	4.54	5.15	0.23	0.01	<0.01	0.22
Glutathione, μmol/g of protein	37.5	59.5	9.54	<0.01	<0.01	0.26
Reduced glutathione, μmol/g of protein	36.4	58.1	9.49	<0.01	<0.01	0.28

¹Ethyl-cellulose rumen-protected methionine effect.

²Ferric-reducing ability of plasma.

³Reactive oxygen metabolites.

Figure 1. Effect of ethyl-cellulose rumen-protected methionine (RPM) supplementation to multiparous Holstein cows from -28 through 60 DIM on plasma biomarkers of liver function (A = cholesterol; B = paraoxonase). Values represent the means $\pm$ SEM. The data from d -30 were included as reference. Treat = treatment.

Biomarkers of Oxidative Stress

Main effects of ethyl-cellulose RPM, time, and interactions on biomarkers of oxidative stress are reported in Table 3. Compared with control, cows supplemented with ethyl-cellulose RPM had greater plasma concentration of FRAP ($P = 0.04$) and lower concentration of ROM ($P = 0.01$; Figure 3). The higher plasma concentration of ROM in the postpartum period was expected because, in conditions of high metabolic demand, the production of free radicals is enhanced partly due to the increase in oxygen requirements (Sordillo and Aitken, 2009). Excessive and rapid production of free radicals can cause an imbalance between ROM concentrations and antioxidant mechanisms, often leading to an increase in oxidative stress. The lower plasma concentration of ROM in cows supplemented with ethyl-cellulose RPM indicated less pronounced oxidative stress status, which agrees with the profiles of inflammation biomarkers.

Figure 2. Effect of ethyl-cellulose rumen-protected methionine (RPM) supplementation to multiparous Holstein cows from -28 through 60 DIM on plasma biomarkers of inflammation (A = albumin; B = haptoglobin; C = IL-1 β ; D = IL-6). Values represent the means $\pm$ SEM. The data from d -30 were included as reference. Treat = treatment.

Compared with controls, cows supplemented with ethyl-cellulose RPM had greater plasma concentrations of β -carotene and tocopherol ($P < 0.02$; Figure 3). These results partly reflect the greater DMI in RPM cows during the postpartum period (Batistel et al.,

2017). The greater concentrations of those vitamins, together with greater plasma concentration of FRAP, is positively associated with antioxidant activity (Benzie and Strain, 1996; Trevisi et al., 2011). Retinol concentration was not different between groups ($P = 0.18$).

Figure 3. Effect of ethyl-cellulose rumen-protected methionine (RPM) supplementation to multiparous Holstein cows from -28 through 60 DIM on plasma and liver biomarkers of oxidative stress [A = ferric-reducing ability of plasma (FRAP); B = reactive oxygen metabolites (ROM); C = β -carotene; D = tocopherol; E = total glutathione; F = reduced glutathione]. Values represent the means $\pm$ SEM. The data from d -30 were included as reference. Treat = treatment.

Table 4. Effects of ethyl-cellulose rumen-protected methionine (RPM) supplementation to multiparous Holstein dairy cows during the close-up (from –28 d to parturition), fresh (from 1 to 30 DIM), and high-producing (from 31 to 60 DIM) periods on blood neutrophil and monocyte function

Item	Control	RPM	SEM	P-value		
				Treatment ¹	Time	Treatment × time
Neutrophils						
Phagocytosis	49.7	59.4	3.07	0.04	0.25	0.77
Oxidative burst	44.6	53.1	3.92	0.06	0.38	0.85
Monocytes						
Phagocytosis	43.9	48.1	2.95	0.12	0.12	0.59
Oxidative burst	25.2	24.5	2.84	0.21	0.22	0.87

¹Ethyl-cellulose rumen-protected methionine effect.

Compared with controls, cows supplemented with ethyl-cellulose RPM had higher concentrations of total glutathione and reduced glutathione in plasma ($P < 0.01$; Figure 3). These data reinforce the conclusion that cows fed ethyl-cellulose RPM experienced less oxidative

stress. Glutathione performs a vital function quenching free radicals and detoxifying xenobiotics (Wu et al., 2004). The GSH produced in liver can be used within the hepatic tissue or exported into the circulation, where it can aid in the control of systemic oxidative stress. Taken together, our results underscore the importance of methionine as a key player in the oxidative stress response, acting as a scavenger of ROM as previously observed in vitro (Campbell et al., 2016), and in dairy cows during the periparturient period (Osorio et al., 2014; Sun et al., 2016; Zhou et al., 2016a).

In Vitro Whole-Blood Phagocytosis and Oxidative Burst

Main effects of ethyl-cellulose RPM, time, and interactions on in vitro whole-blood phagocytosis and oxidative burst are reported in Table 4. Upon in vitro *Escherichia coli* challenge, a greater ($P = 0.04$) increase in neutrophil phagocytosis capacity was detected in cows supplemented with ethyl-cellulose RPM (Table 4, Figure 4). Furthermore, compared with control cows, ethyl-cellulose RPM-supplemented cows tended to have greater blood neutrophil oxidative burst ($P = 0.06$). In contrast, monocyte phagocytosis and oxidative burst did not change in response to ethyl-cellulose RPM supplementation ($P \geq 0.12$). Neutrophils and macrophages are the first line of defense of the innate immune system by phagocytosing, killing, and digesting invading pathogens. Because the activation of the immune system is an energy-dependent process, the greater DMI in ethyl-cellulose RPM (Batistel et al., 2017) could have contributed, at least in part, to the observed response in neutrophils. Furthermore, the lower concentrations of myeloperoxidase in ethyl-cellulose RPM cows could sustain neutrophil function, as oxidative stress is detrimental to immune cells (Spears and Weiss, 2008).

Little evidence exists demonstrating that methionine per se alters immune function. However, the biologic

Figure 4. Effect of ethyl-cellulose rumen-protected methionine (RPM) supplementation to multiparous Holstein cows from –28 through 60 DIM on neutrophil phagocytosis (A) and oxidative burst (B). Values represent the means $\pm$ SEM.

effect on immune function from the total quantity of Met supplied is supported by the immunomodulatory capacity of its metabolites (taurine, GSH, and homocysteine; Grimbale, 2006). For instance, in vivo models have demonstrated that taurine supplementation enhanced neutrophil phagocytic and bactericidal capacity (Masuda et al., 1984), whereas GSH helped sustain the oxidative burst response (Yan et al., 2012). Taken together, the present and previous data (Zhou et al., 2016a) underscore the role of Met on neutrophil function in periparturient cows.

CONCLUSIONS

Overall, the changes observed in plasma biomarkers indicate that feeding ethyl-cellulose RPM to achieve a ratio of 2.8:1 Lys to Met during the periparturient period and early lactation is an effective approach to help mitigate oxidative stress and inflammation as well as enhance liver and neutrophil function. Our results indicated that the positive effect on postpartal cow performance was likely due, at least in part, to a better immune response. Additional studies to evaluate this potential effect are warranted.

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