Bovine Neutrophil Responses to Parenteral Vitamin E[1]

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ABSTRACT

Twenty-eight Holsteins were tested to determine effects of dietary and parenteral vitamin E supplementation during the dry period on plasma α-tocopherol and in vitro neutrophil functions at calving. Cows were assigned to one of four experimental groups receiving either supplemental dietary vitamin E, injections of vitamin E, both dietary and injections of vitamin E, or neither source of supplemental vitamin E during the dry period in a 2 × 2 factorial arrangement. Cows receiving parenteral vitamin E were injected subcutaneously with 3000 IU of vitamin E (dl-α-tocopherol) at 10 and 5 d prior to anticipated calving. Cows not receiving parenteral vitamin E were injected with a placebo. Experimental groups receiving dietary vitamin E during the dry period were supplemented with 1040 IU/d compared with none for controls. Cows injected with vitamin E had greater plasma α-tocopherol concentration 5 d after the first injection, at calving, and 1 wk after calving than did cows injected with placebo. Plasma α-tocopherol concentrations did not differ between dietary vitamin E treatment groups from calving through 4 wk postpartum. No interaction was found between dietary and parenteral supplementation of vitamin E on plasma α-tocopherol concentration. Neutrophils from cows injected with vitamin E had greater intracellular kill of bacteria at calving than did neutrophils from placebo-injected cows. Neither phagocytic index nor percentage of neutrophils phagocytizing differed between vitamin E-injected and placebo-injected cows. Dietary vitamin E during the dry period had no effect on neutrophil function at calving. Intracellular kill and plasma α-tocopherol were correlated at calving.
(**Key words**: vitamin E, neutrophils, parenteral)

Abbreviation key: **HBSS** = Hanks balanced salt solution.

INTRODUCTION

Dietary deficiencies in vitamin E were associated with increased prevalence of mastitis (13). Plasma vitamin E concentrations in dairy cows are normally lowest when rates of IMI are highest and when neutrophil functions are depressed. Rates of IMI and clinical mastitis are highest during the first 7 d after calving compared with other stages of lactation (12, 21). The high rate of new IMI at calving coincides with reported immunosuppression and decreased bactericidal activity by neutrophils during wk 1 of lactation (15). Plasma vitamin E concentrations in multiparous cows also decrease approximately 7 d prior to calving and remain depressed for 7 to 14 d after calving (22, 23). The relationship among these occurrences may be that vitamin E is critical in protecting neutrophils from the destructive action of toxic oxygen molecules necessary for intracellular kill of ingested pathogens (1, 14, 18).

Decreased plasma α-tocopherol during the periparturient period is due in part to decreased feed intake during this period. Plasma α-tocopherol concentrations are sensitive to changes in consumption of vitamin E in dairy

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cows. Concentration of plasma α-tocopherol and dietary intake both decreased during the periparturient period even though the amount of dietary vitamin E offered to animals was constant throughout this period (22). Administration of vitamin E to late gestation cows in a manner other than in feed may maintain plasma concentrations of vitamin E. Parenteral administration of vitamin E elevated plasma vitamin E successfully during early lactation (11). The purpose of this study was to determine whether injections of vitamin E prior to calving maintain α-tocopherol concentration in plasma and prevent suppression of neutrophil function at calving.

MATERIALS AND METHODS

Experimental Design

Twenty-eight Holsteins in the Ohio Agricultural Research and Development Center herd were assigned to one of four experimental groups balanced by parity, milk production in the previous lactation, and IMI status at drying off. Cows were dried off 60 d before anticipated calving by abrupt cessation of milking, and all four quarters were infused with a dry cow antibiotic product containing 300 mg of cephapirin (Tomorrow, Franklin Laboratories, Amarillo, TX).

Experimental groups received either supplemental dietary vitamin E (n = 7), injections of vitamin E (n = 9), both dietary and injected vitamin E (n = 6), or neither source of supplemental vitamin E (n = 6) during the dry period in a 2 × 2 factorial arrangement. Only cows that calved within ± 3 d of anticipated calving were used. Cows receiving parenteral vitamin E were injected (total 12 ml) with 3000 IU of vitamin E (dl-α-tocopherol) in a 20% ethyl alcohol and 1% benzyl alcohol emulsifiable solution (Rocavit-E, Hoffman-LaRoche, Nutley, NJ) at 10 and 5 d prior to anticipated calving. Cows not receiving parenteral vitamin E were injected at 10 and 5 d prior to anticipated calving with a placebo (12 ml) containing the alcohol emulsifiable solution void of vitamin E. Injections were subcutaneous on the upper part of the rib cage just posterior to the scapula.

Dry cows were fed approximately 6 kg of late vegetative grass silage, .9 kg of concentrate, and 3.5 kg of mature grass hay daily (DM basis). The concentrate mix was 96.5% corn, 1.5% limestone, 1.5% trace-mineralized salt, and .5% vitamin premix. Each kilogram of concentrate provided 60,000 IU of vitamin A and 20,000 IU of vitamin D. All cows received a basal amount of 300 IU/d of vitamin E. Rations for the two experimental groups receiving dietary vitamin E during the dry period were supplemented with an additional 1040 IU/d. Dry cow diets were adequate in all other nutrients, including .3 ppm of selenium. All cows were fed the same diet following parturition. The lactating cow diet was adequate as specified by the NRC (16), including .3 ppm of selenium and 500 mg of supplemental vitamin E. Feeds were analyzed monthly for α-tocopherol and α-tocopherol acetate (23). Cows were group fed during the dry period until 10 d prior to anticipated calving. Cows were fed individually from 10 d prior to calving until 4 wk after calving.

Blood samples were collected from all cows at drying off, 30 d after drying off, 10 d and 5 d prior to anticipated calving, within 48 h after calving, and 1, 2, and 4 wk postpartum to determine α-tocopherol concentrations. Samples 10 and 5 d prior to calving were collected before cows received injections. Plasma was collected, and α-tocopherol was analyzed as described previously (14, 23). Glutathione peroxidase activity (17) was measured from whole blood samples collected at drying off, calving, and 4 wk into lactation.

Neutrophil Assay

Blood samples were collected from all cows within 48 h after calving and 1, 2, and 4 wk into lactation to determine in vitro neutrophil function. Blood samples were collected as described by Carlson and Kaneko (6). Final cell preparations were washed twice in Hanks balanced salt solution (HBSS; pH 7.2). Viable cells were determined by trypan blue exclusion and counted with a hemocytometer. A portion of each final cell preparation was stained for differential counts (Diff-Quik; AHS del Caribe, Inc., Aguada, Puerto Rico). Cell preparations averaged ($\overline{X}$ ± SD) 94.6 ± 2.8% neutrophils and 95.9 ± 1.4% viability. Cell concentrations were adjusted to 25 × 10^6 viable neutrophils/ml of HBSS.

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Bacteria tested were *Escherichia coli* (McDonald 487). *Escherichia coli* 487 is a strain originally isolated from a clinical case of bovine mastitis. Prior to testing, bacteria were stored in trypticase soy broth containing 20% glycerin at –70°C. A total of .1 ml of thawed stock culture was inoculated into 12 ml of trypticase soy broth and incubated overnight at 37°C on a gyratory shaker at 200 rpm. A .2-ml portion of the overnight culture was inoculated into 24 ml of fresh trypticase soy broth and incubated for 2.5 h at 37°C and 50 rpm. Bacteria were centrifuged and resuspended in HBSS. Bacterial cultures were diluted in HBSS to 70% transmission at 540 nm (Beckman DU-50 Spectrophotometer, Beckman Instruments, Fullerton, CA). Bacteria were opsonized in 20% serum for 20 min at 20°C. Serum for opsonization was collected from 9 lactating cows, pooled, and heated to 56°C for 30 min to inactivate complement. Bacteria were diluted to approximately 75×10^6 cfu/ml.

Phagocytosis and intracellular kill of bacteria by neutrophils were measured by modifications of the fluorochrome assay described by Goldner et al. (9). Briefly, suspensions of neutrophils and opsonized bacteria were added to incubation tubes in a ratio of 3:1 (bacteria: neutrophils) and incubated at 37°C at 100 rpm on a gyratory shaker for 90 min. Bacterial numbers were confirmed by removing a portion of assay suspension prior to incubation, serially diluting bacteria, and plating bacteria on trypticase soy agar. Following incubation, samples were removed and diluted 2:1:1 as assay suspension:acridine orange (14 mg/100 ml of PBS):crystal violet (50 mg/100 ml of PBS). Wet mount slides were prepared, and the number of live (green bacterial cells) and dead (red bacterial cells) bacteria were counted in the first 50 neutrophils visible under 1000 × oil immersion magnification while the stage of the fluorescence microscope (Nikon Fluorescence Microscope, Nikon Inc., Garden City, NJ) was moved horizontally from the left to right edges of the cover slip. Phagocytic index was calculated as average number of bacteria phagocytosed per neutrophil. Intracellular kill was determined as (number of dead phagocytosed bacteria/number of live + number of dead intracellular bacteria × 100). Percentage of neutrophils phagocytizing was calculated. All assays were in duplicate and were conducted blind: laboratory personnel did not have prior knowledge of cow, day of lactation, or experimental group identification.

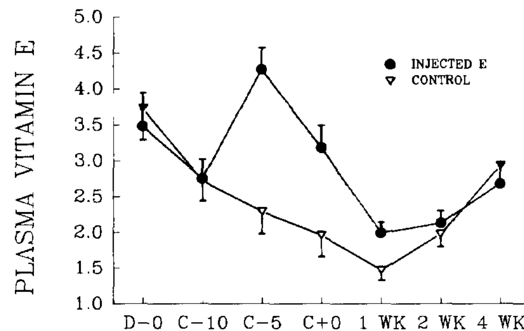

Figure 1. Mean (± SE) plasma concentrations of vitamin E in cows injected either with 3000 IU of vitamin E (n = 15) or placebo (n = 13) at 10 and 5 d prior to anticipated calving. Samples were collected at drying off (D-0); 10 d (C-10) and 5 d (C-5) prior to anticipated calving; within 48 h after calving (C+0); and 1, 2, and 4 wk after calving.

Statistical Analyses

Plasma α-tocopherol, whole blood glutathione peroxidase, and neutrophil function data were analyzed using least squares analysis of variance. Models included the main effects of injection, diet, and the interaction between injection and diet. Ratio of bacteria:neutrophil in each neutrophil assay was unrelated to treatment but was a significant ($P < .05$) effect on phagocytic index and intracellular kill. Ratio of bacteria:neutrophil was included as a covariate to adjust for neutrophil assay variability unrelated to treatment. Data were analyzed within sample period. Relationships among plasma concentrations of α-tocopherol and in vitro neutrophil functions were quantified using linear and multiple regression (20).

RESULTS

Cows injected with vitamin E had greater plasma α-tocopherol concentration 5 d after the first injection, at calving, and 1 wk after calving than did cows injected with placebo (Figure 1; $P < .05$). Differences between plasma α-tocopherol concentrations in vitamin E and placebo-injected cows were not significant 2 and 4 wk after calving. Cows fed diets

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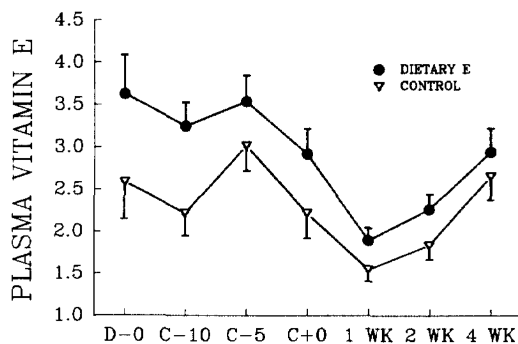

Figure 2. Mean (± SE) plasma concentrations of vitamin E in cows fed diets either supplemented (n = 13) or unsupplemented (n = 15) with vitamin E during the dry period. Samples were collected at drying off (D-0); 10 d (C-10) and 5 d (C-5) prior to anticipated calving; within 48 h after calving (C+0); and 1, 2, and 4 wk after calving.

supplemented with vitamin E during the dry period had greater plasma α-tocopherol 10 d prior to anticipated calving than did cows fed unsupplemented diets (Figure 2; $P < .05$). Plasma α-tocopherol did not differ between dietary vitamin E treatment groups at 5 d prior to anticipated calving, at calving, or during early lactation. No interaction was found between dietary and parenteral supplementation of vitamin E on plasma α-tocopherol concentration. Whole blood glutathione peroxidase was adequate in all cows and unrelated to either dietary or parenteral vitamin E ($P > .05$). Mean (± SD) glutathione peroxidase (millienzyme units per milligram of hemoglobin) among all cows was 77.8 ± 15.1, 79.5 ± 12.2, and 75.2 ± 13.7 at drying off, calving, and 4 wk into lactation, respectively.

Cows injected with vitamin E had greater intracellular kill of bacteria at calving than did placebo-injected cows ($P < .05$). Mean (± SE) intracellular kill was 80.1 ± 3.1 for vitamin E-injected cows and 70.8 ± 4.4 for placebo-injected cows (Figure 3). Differences in intracellular kill between injection groups were not significant at 1, 2, and 4 wk after calving. Intracellular kill was greater at wk 2 and 4 of lactation than at calving within placebo-injected cows ($P < .05$). Intracellular kill did not differ among sampling periods within vitamin E-injected cows. Neither phagocytic index (Figure 4) nor percentage of neutrophils phagocytizing (Figure 5) differed between vitamin E-injected and placebo-injected cows. Cows fed supplemental vitamin E during the dry period had greater intracellular kill at 4 wk after calving than cows fed control diets (Figure 6). Intracellular kill did not differ between dietary groups at either calving, 1 wk, or

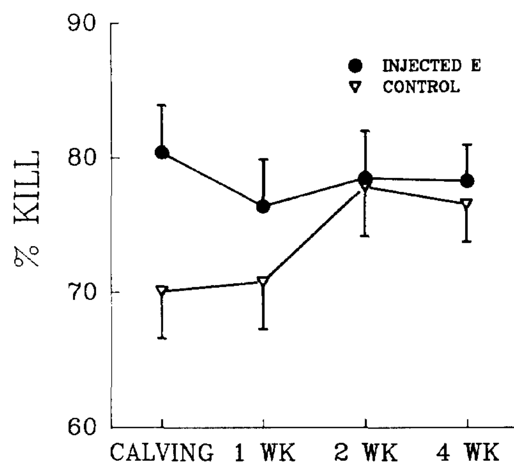

Figure 3. Mean (± SE) in vitro intracellular kill of bacteria by neutrophils isolated from cows injected with either 3000 IU of vitamin E (n = 15) or placebo (n = 13) 10 and 5 d prior to calving. Neutrophils were collected within 48 h after calving and 1, 2, and 4 wk after calving.

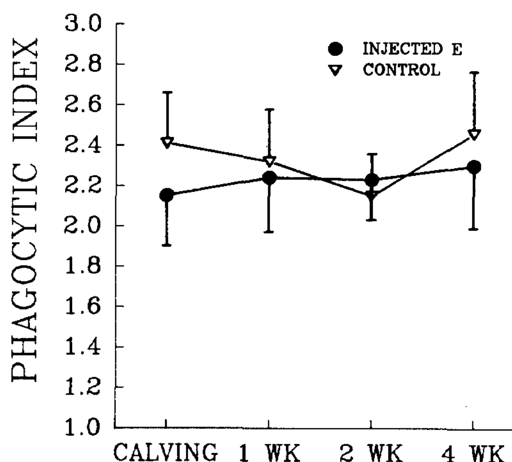

Figure 4. Mean (± SE) phagocytic index of neutrophils isolated from cows injected with either 3000 IU of vitamin E (n = 15) or placebo (n = 13) 10 and 5 d prior to calving. Neutrophils were collected within 48 h after calving and 1, 2, and 4 wk after calving.

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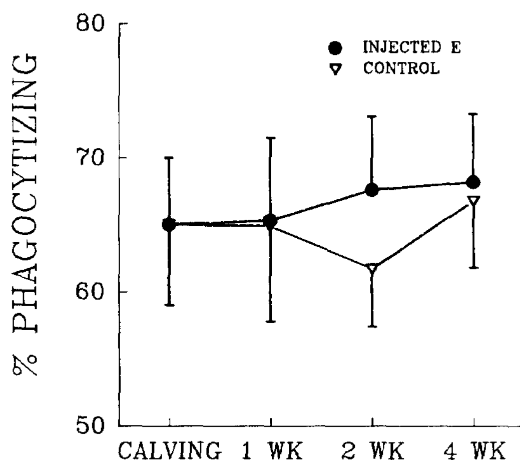

Figure 5. Mean (± SE) percentage of neutrophils phagocytizing from cows injected with either 3000 IU of vitamin E (n = 15) or placebo (n = 13) 10 and 5 d prior to calving. Neutrophils were collected within 48 h after calving and 1, 2, and 4 wk after calving.

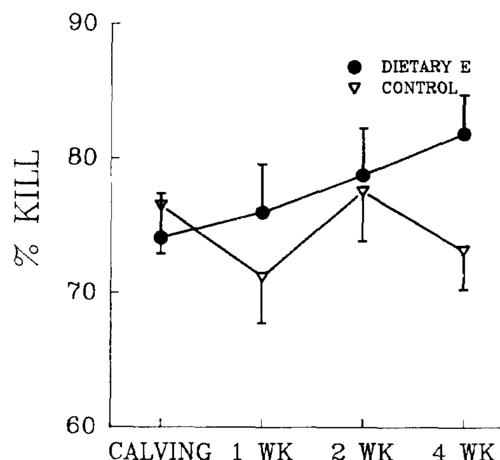

Figure 6. Mean (± SE) in vitro intracellular kill of bacteria by neutrophils isolated from cows fed dry period diets either supplemented (n = 13) or unsupplemented (n = 15) with vitamin E. Neutrophils were collected within 48 h after calving and 1, 2, and 4 wk after calving.

2 wk after calving. Dietary vitamin E treatments had no effect on either phagocytic index (Figure 7) or percentage of neutrophils phagocytizing (Figure 8). No interactions were found between dietary and parenteral supplementation of vitamin E on neutrophil functions.

The correlation coefficient between intracellular kill and plasma α-tocopherol was r = .41 at calving ($P < .05$). Correlation coefficients between intracellular kill and plasma α-tocopherol at wk 1, 2, and 4 of lactation were not significant. Phagocytic index and percentage of neutrophils phagocytizing were not correlated with plasma α-tocopherol.

DISCUSSION

Plasma α-tocopherol concentrations decrease dramatically during the periparturient period and may be sensitive to changes in consumption of vitamin E in dairy cows (22, 23). Concentration of plasma α-tocopherol typically drops by approximately 50% and remains low during the first weeks of lactation, even when dietary vitamin E offered to cows is constant throughout this period. Likewise, dietary supplementation of vitamin E during the dry period did not maintain plasma α-tocopherol at calving and during the first weeks of lactation in the present study. Administration of vitamin E to late gestation cows in a manner other than in feed was tested as a means to maintain plasma concentrations of vitamin E. Parenteral administration of vitamin E successfully elevated plasma α-tocopherol during late gestation and early lactation periods.

Vitamin E enhances host defenses against infections by improving phagocytic cell function. Vitamin E is an antioxidant that protects phagocytic cells and surrounding tissues from oxidative attack by free radicals produced by the respiratory burst of neutrophils and macrophages during phagocytosis (1, 2, 3). The respiratory burst by neutrophils is characterized by marked changes in oxygen metabolism that result in increased production of superoxide and hydrogen peroxide (1). Vitamin E is localized in cellular membranes in close proximity to oxidase enzymes that initiate the production of free radicals. Polyunsaturated fatty acids located in the vicinity of the oxidase enzymes are protected from peroxidation by vitamin E (2). Vitamin E inhibits autoxidation of polyunsaturated fatty acids in neutrophil membranes (4, 5) and enhances neutrophil function (2). Impaired neutrophil function in cows during

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