Peripartum Serum Vitamin E, Retinol, and Beta-Carotene in Dairy Cattle and Their Associations with Disease

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ABSTRACT

Peripartum decreases in serum concentrations of vitamins A and E may contribute to impaired immune function in dairy cows. The objectives of this study were to describe peripartum serum concentrations of α -tocopherol, β -carotene, and retinol and their associations with disease risk. On 20 farms over 1 yr, blood samples were collected weekly from 1057 cows from 1 wk before expected calving until 1 wk postpartum. Serum concentrations of α -tocopherol, β -carotene, and retinol, as well as several biochemical variables were measured. Their associations with the risk of retained placenta or clinical mastitis were modeled separately with logistic regression, and the factors associated with the concentration of each vitamin were modelled with mixed linear regression. Differences in vitamin concentrations between 2 batches of sera analyzed 6 mo apart required stratification of statistical analyses. Accounting for the effects of parity, season, and twins, an increase in α -tocopherol of 1 μ g/mL in the last week prepartum reduced the risk of retained placenta by 20%, whereas serum nonesterified fatty acid concentration ≥ 0.5 mEq/L tended to increase risk of retained placenta by 80%. In the last week prepartum, a 100 ng/mL increase in serum retinol was associated with a 60% decrease in the risk of early lactation clinical mastitis. There were significant positive associations of peripartum serum concentrations among each of α -tocopherol, β -carotene, and retinol.

(Key words: vitamin A, vitamin E, retained placenta, mastitis)

Abbreviation key: RP = retained placenta.

INTRODUCTION

The transition period for dairy cows is characterized by increased risk of several metabolic and infectious

diseases. One important causal factor is impaired immune function in peripartum cows (Mallard et al., 1998), and cows' vitamin A and vitamin E status are component factors in immune function (NRC, 2001). Peripartum immunosuppression is multifactorial but is associated with endocrine changes and decreased intake of critical nutrients (Goff and Horst, 1997). Circulating concentrations of vitamins A and E decrease around calving (Goff et al., 2002). Decreased phagocytosis and intracellular killing by neutrophils occur in parallel with decreased DMI and decreased circulating vitamin E (α -tocopherol) concentration (Hogan et al., 1992). Vitamin E is a fat-soluble membrane antioxidant that enhances the functional efficiency of neutrophils by protecting them from oxidative damage following intracellular killing of ingested bacteria (Herdt and Stowe, 1991). Several studies (Weiss et al., 1990; Weiss et al., 1992) have shown that dietary supplementation with 1000 IU of vitamin E per cow per day in the late dry period mitigates the peripartum drop in circulating α -tocopherol, but this does not necessarily decrease the incidence of disease (Allison and Laven, 2000). Beta-carotene is the main dietary precursor of vitamin A (retinol) in dairy cattle. Beta-carotene that escapes rumen degradation is metabolized in the intestinal mucosa to retinol and absorbed and transported to the liver with fat (Chew, 1987). Vitamin A has numerous functions that are not fully understood. In peripartum dairy cows, it is reported to have a role in resistance to infectious disease, particularly mastitis (NRC, 2001). In dairy cattle, β -carotene may also exert an effect as an antioxidant, separate from its role as provitamin A (Chew 1993).

The study reported here is derived retrospectively from samples collected from a previously reported clinical trial conducted near Guelph, Ontario, Canada, using 1142 Holstein cows from 20 herds (LeBlanc et al., 2002). The objective of the previous clinical trial was to determine the effect of one subcutaneous injection of 3000 IU of vitamin E 1 wk before expected calving on the incidence of retained placenta (RP). The data collected for that trial provided a large dataset, including disease incidence and metabolic profiles, as well as a bank of

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sera. This afforded an opportunity to intensively study the associations between serum measures of vitamin A and vitamin E status and health at the individual cow level in a much larger sample than would normally be available.

The objectives of this study were to describe factors affecting the serum concentrations of α -tocopherol, β -carotene, and retinol during the periparturient period, to assess their association with the occurrence of periparturient disease, and to define thresholds for disease risk.

MATERIALS AND METHODS

Details of the clinical trial, data collection, laboratory and statistical analyses, and overall disease incidences were reported in LeBlanc et al. (2002). Briefly, from September 1998 through October 1999, a research technician visited each of 20 farms weekly on the same day, at approximately the same time, within 2 h of the morning feeding. All cows were randomly assigned to receive either 3000 IU of RRR- α -tocopheryl acetate (label dose of Vital-E 300, Schering-Plough, Union, NJ) s.c. or a placebo (propylene glycol), once, at 1 wk before expected calving. This dose has been reported to decrease the incidence of RP (Erskine et al., 1997) and to increase serum α -tocopherol for approximately 2 wk (Erskine et al., 1997; LeBlanc et al., 2002). Blood samples were collected at the time of treatment (4 to 10 d before expected calving), and once weekly thereafter until the first week postpartum. Serum was harvested and an aliquot stored at -20°C within 5 h of collection. Serum biochemistry analyses were conducted at the Animal Health Laboratory, University of Guelph including concentrations of BHBA, NEFA, cholesterol, glucose, urea, calcium, and phosphorus. Cows' body condition was scored at enrollment, and disease events (for case definitions, see LeBlanc et al., 2002) were intensively recorded for 1 mo after calving. The case definition for RP was failure to pass the fetal membranes by 24 h after calving, and for clinical mastitis was producer diagnosis of abnormal milk or swelling of the udder, including cows with systemic illness attributed to mastitis, within 30 d after calving.

All serum vitamin analyses were performed at the Animal Health Diagnostic Laboratory of Michigan State University (MSU) by a standard HPLC method for simultaneous measurement of α -tocopherol, β -carotene, and retinol concentrations (modification of Arnaud et al., 1991). Samples were processed 50 at a time, and the intraassay and interassay coefficients of variability were 16.7 and 13.1%, respectively. The sera were shipped frozen and on ice by overnight courier from Guelph to East Lansing in 2 batches. The initial

submission (batch 1; $n = 785$ samples from 310 cows) in January 2001 consisted of all serum samples from cows that had RP (358 samples from 145 cows in 20 herds), and all samples from a similar number of randomly selected cows that did not have RP (427 samples from 165 cows in 19 herds), and was analyzed within 1 mo. Subsequently, all remaining samples from all cows (none of which had a recorded RP) were shipped to MSU in May 2001 (Batch 2; $n = 1829$ samples from 747 cows). These samples were processed over the remainder of that year, until December 2001.

All statistical analyses were performed with SAS version 8.0 (SAS Institute, 1999, Cary, NC). The means for each vitamin in each batch were compared with the t -test statistic (Proc Ttest). Because there were significant differences between batches, the data from each batch were modelled separately. Determinants of risk of RP and clinical mastitis before 30 DIM were modelled in batches 1 and 2, respectively, with multivariable logistic regression, accounting for clustering of cows within herds with generalized estimating equations (Proc Genmod, with binary distribution, logit link function, and compound symmetry correlation structure; Shoukri and Pause, 1999). In the disease models, vitamin concentrations as model inputs were treated as continuous variables.

Considering serum vitamin concentration as the outcome of interest, the cow was the unit of interest, with 2 to 4 repeated measures. Vitamin E is transported in serum in lipoproteins. To account for changes in transport capacity in transition cows that are mobilizing fat, vitamin E is also expressed as α -tocopherol:cholesterol mass ratio (Weiss et al., 1992; Herdt and Smith, 1996). Each of α -tocopherol, β -carotene, and retinol concentrations, and α -tocopherol:cholesterol mass ratio were modeled separately with multivariable linear regression, accounting for repeated measures of the outcome within a cow, and the clustering of cows within herds (Proc Mixed, with autoregressive correlation structure and random effect of herd).

For both sets of models, all available variables, including cow factors (parity, season of calving, BCS, and vitamin E treatment in the clinical trial), disease events, and metabolic variables (serum NEFA, BHB, glucose, cholesterol, urea, calcium, and phosphorus concentrations) were offered initially. Models were reduced by manual backward stepwise regression until all main effects were significant at $P < 0.1$.

RESULTS

Samples drawn more than 16 d before actual calving were excluded from the analysis because of the sparseness of those data.

Table 1. Serum vitamin concentrations in peripartum Holstein cows around Guelph, Ontario, Canada, over 1 yr.¹

Vitamin	Batch 1			Batch 2			Mean difference	SD of difference	P
	n	Mean	SD	n	Mean	SD			
α -Tocopherol ($\mu\text{g/mL}$)	785	2.71	1.38	1829	2.06	1.10	-0.69	1.19	<0.0001
α -Tocopherol: cholesterol ($\times 10^{-3}$)	778	3.44	1.78	1813	2.54	1.43	-0.91	1.54	<0.0001
β -Carotene ($\mu\text{g/mL}$)	748	1.52	1.18	1828	1.12	0.78	-0.40	0.91	<0.0001
Retinol (ng/mL)	749	149	73	1828	191	76	42	75	<0.0001

¹Samples were collected and immediately frozen between September 1998 and October 1999, then shipped frozen and on ice by overnight courier from Guelph to East Lansing in 2 batches: Batch 1 shipped in January 2001, analyzed in February 2001 and Batch 2, shipped in May 2001, analyzed between May and December 2001.

The mean serum vitamin concentrations and the significant differences between batches are presented in Table 1. Samples analyzed in batch 2, on average, had concentrations of α -tocopherol, β -carotene, and retinol that were 0.7 and 0.4 $\mu\text{g/mL}$ lower, and 42 ng/mL higher than in batch 1, respectively. Because of this effect, all subsequent analyses were stratified on batch.

Figures 1 and 2 provide descriptive information on the peripartum changes in circulating concentrations of vitamin E, β -carotene, and retinol. Figure 1 illustrates vitamin concentrations in batch 1 in cows with and without RP. Using a separate subset of the data (Batch 2), Figure 2 shows the serum vitamin concentrations in cows with and without early lactation clinical mastitis. Among cows that experienced an episode of clinical mastitis before 30 DIM, the median time of occurrence was 2 DIM. Note that because of the serum batch effect, the absolute values of vitamin concentrations in Figures 1 and 2 are not comparable.

Samples were collected starting, on average, 7 d before calving occurred (range 0 to 16 d). For models of disease occurrence (Tables 2 to 4), data from the second week prepartum were too sparse for multivariable models to converge. Therefore, serum samples taken in the last week before calving (mean ($\pm$ SD) and median time to calving = 3.0 ± 2.0 d prepartum; range of 6 d prepartum to just before parturition) were examined for association with RP (Table 2). Because samples were collected weekly, only one sample per cow was included in the model. Accounting for the effects of parity, season, and twin birth, for every 1 $\mu\text{g/mL}$ increase in serum vitamin E concentration the risk of RP decreased by 21%. Cows with NEFA ≥ 0.5 mEq/L in the last week prepartum tended ($P = 0.07$) to be 77% more likely to experience RP. In a separate model (not shown) in which vitamin E was expressed as α -tocopherol:cholesterol mass ratio, the final variables and their parameter estimates were very similar to those in Table 2. For each 1 unit increase in α -tocopherol:cholesterol mass ratio, the probability of RP was reduced by 17% (odds ratio = 0.83, 95% confidence interval 0.68 to 1.01, $P = 0.06$).

Table 3 presents measures of the association of various cut-points of vitamin E in the last 7 d prepartum with the risk of RP, adjusted for the other significant variables in Table 2. Cows with α -tocopherol:cholesterol mass ratio $< 2.5 \times 10^{-3}$ tended ($P = 0.07$) to have almost twice the probability of having RP.

As for the RP model, samples (one per cow, taken between 6 d before calving and the day of calving) were examined as determinants of the risk of clinical mastitis (Table 4). The preponderance of recorded cases occurred at or soon after calving (median time to diagnosis was 2 d after calving). In this time frame, an increase in serum retinol concentration of 100 ng/mL was associated with a 60% reduction in the risk of mastitis ($P = 0.001$). There was also a tendency ($P = 0.1$) for increased cholesterol concentration to increase the risk of mastitis by 80%.

With stratification on batch, there were too few cases of metritis or displaced abomasum for multivariable models that included vitamin concentrations to converge. Likewise, clinical mastitis could not be modeled in batch 1, because among 138 cows there were too few cases ($n = 19$) for models containing all independent variables to converge.

Factors affecting the peripartum concentration of vitamin E were modeled separately for each batch of sera (Tables 5 and 6). Although the absolute vitamin concentrations are not comparable between batches, the magnitudes of effects within the models are comparable. There was generally good agreement between the two models. Accounting for all the other variables in the final models one injection of 3000 IU of RRR-tocopheryl acetate s.c. 1 wk before expected calving raised average serum α -tocopherol concentration by 0.4 to 0.5 $\mu\text{g/mL}$ from injection until the first week postpartum. Peripartum serum α -tocopherol concentrations were generally highest in the summer. Serum cholesterol concentration was positively associated with serum α -tocopherol concentration. Serum concentrations of β -carotene and retinol were positively associated with serum α -tocopherol concentrations. The covariance parameter estimates indicated that intra-herd correlation of α -tocoph-

Figure 1. Peripartum serum vitamin concentrations (mean and SE) in cows with and without retained placenta (RP). Samples were from the approximately one-half of cows in batch 1 that did not receive an injection of vitamin E (67 cows with RP and 71 cows without RP). Each cow was sampled once weekly. Data are pooled into 2-d increments relative to actual day of calving.

erol concentrations was low, whereas intra-cow correlation was low to moderate, depending on the model. In other words, there was equal or greater variance in serum α -tocopherol among cows within a herd, as there was variance between herds. Overall, both these sources of variance were small relative to other, unmeasured sources (residual error).

Tables 7 and 8 describe factors affecting peripartum β -carotene concentration in each batch of sera. Accounting for all the other variables in the final models, mature cows had slightly higher serum β -carotene than animals in first or second parity. Serum β -carotene was 0.3 to 0.5 $\mu\text{g/mL}$ higher in summer than in any other season. There were significant positive associations of both α -tocopherol and retinol with β -carotene concentrations. On average, a 1 $\mu\text{g/mL}$ increase in α -tocopherol was associated with a 0.2 to 0.3 $\mu\text{g/mL}$ increase in se-

rum β -carotene, while a 100 ng/mL increase in retinol was associated with a 0.2 to 0.4 $\mu\text{g/mL}$ increase in serum β -carotene. The covariance parameter estimates were consistent between the two models, and indicated that the effects of within-herd and within-cow correlation explained over half the variance in the data. Intra-cow correlation was 3- to 4-fold that of herd clustering, indicating that there was substantially more variability among individual cows than among herds.

The factors associated with peripartum serum retinol concentration were modeled for each batch of sera (Tables 9 and 10). Accounting for all the other variables in the final models, mature cows had slightly lower retinol concentrations than first- and second-lactation animals, retinol concentrations were lowest in the summer and highest in the autumn, and there was a positive association between circulating concentrations of β -car-

Figure 2. Peripartum serum vitamin concentrations (mean and SE) in cows with and without clinical mastitis before 30 DIM. Batch 2. Each cow was sampled once weekly. Data are pooled into 2-d increments relative to actual day of calving.

Table 2. Logistic regression model of factors measured in the last week prepartum associated with the risk of retained placenta (batch 1, $n = 138$ cows all untreated with vitamin E injection; 67 RP, 71 controls; 1 sample per cow). Vitamin E expressed as serum α -tocopherol concentration.

Variable	Level	Parameter estimate	Robust SE ¹	OR ²	95% CI ³	P
Intercept		0.639	0.537	...	...	...
Parity group	1	-0.736	0.315	0.47	0.26–0.89	0.02
	2	-1.178	0.419	0.31	0.14–0.70	0.005
	≥ 3	Referent	...	...	...	...
Season of calving	Sep–Nov	0.380	0.423	1.46	0.63–3.35	0.37
	Dec–Feb	0.373	0.472	1.45	0.58–3.63	0.43
	Mar–May	0.188	0.686	1.21	0.31–4.63	0.78
	Jun–Aug	Referent	...	...	...	...
Twins	yes	1.352	0.886	3.86	0.68–21.9	0.12
Serum NEFA	≥ 0.5 mEq/L	0.574	0.320	1.77	0.95–3.32	0.07
α -Tocopherol	Per 1 $\mu\text{g/mL}$ increase	-0.233	0.097	0.79	0.66–0.96	0.02

¹Correlation of cows within herd accounted for with generalized estimating equations.

²OR = Odds ratio.

³95 CI = 95% confidence interval.

Table 3. Thresholds of least squares means of measures of serum vitamin E in the last week prepartum for risk of retained placenta, adjusted for the effects of parity, herd, season, twins, and serum NEFA. Based on Table 2 and a separate model (not shown) where vitamin E was expressed as α -tocopherol:cholesterol mass ratio, in which the final covariates and their estimates were very similar.

Measure and threshold	Odds ratio	P
α -Tocopherol:cholesterol mass ratio		
$<2.0 \times 10^{-3}$	3.1	0.002
$<2.5 \times 10^{-3}$	1.9	0.07
α -Tocopherol		
$<2.0 \mu\text{g/mL}$	1.5	0.16
$<2.5 \mu\text{g/mL}$	1.2	0.18
$<3.0 \mu\text{g/mL}$	1.1	0.62

otene and retinol. There was also a significant, but smaller per unit, association between serum concentrations of α -tocopherol and retinol. Serum retinol concentration was significantly positively associated with serum calcium concentration, and inversely associated with NEFA concentration. The covariance parameter estimate indicated that there was essentially no intracow correlation, and low to moderate intraherd correlation. Most (85 to 95%) of the variance in serum retinol was attributable to unmeasured sources.

DISCUSSION

The significant and unaccountable differences in vitamin concentrations between the two batches of sera reduced the statistical power of this investigation. Notwithstanding this handicap, these datasets still represent useful information, which even separately are large for the breadth of variables and frequency of sampling that they contain on peripartum dairy cows.

It was expected that the two batches would yield similar overall values for vitamin concentrations. Batch 1, containing cows with RP and randomly selected cows without RP, may have been expected to have lower mean values for α -tocopherol, because low circulating concentration of vitamin E has been shown to be a risk factor for RP (LeBlanc et al., 2002), and this subset of sera contained all cows with RP. One approach would be to add an adjustment factor to the values from one batch, to make them comparable to the other. Such an approach requires assumptions that it is known which values are correct and which require adjustment, and that the adjustment factor is constant within the batch, or within specific strata within the batch. Based on expected values, it appeared that the results for α -tocopherol and β -carotene from Batch 2 were suspiciously low. Unfortunately, there was no biological or statistical means to verify this assumption in this case.

The biological basis for the batch effect was not clear. The possibilities include decay of α -tocopherol and β -carotene in frozen storage (although all samples were frozen for at least 1 yr, with batch 2 frozen 6 mo longer than batch 1), degradation in shipping or handling (e.g. thaw and refreeze, or heating), or a change or artifact in the laboratory measurement itself (none known). The effect of frozen storage of bovine sera for more than 1 wk on α -tocopherol is not known (Mitsioulis and Judson, 2000). Based on exclusion of known factors, it seems most likely that the samples in batch 2 were affected by conditions in transit. Given this effect, and the uncertainty of the validity of applying a constant adjustment value to the results, all analyses were stratified by batch. Because of the way the batches were assembled, determinants of RP were modelled in batch 1 only, and determinants of mastitis were modeled in batch 2 only.

Table 4. Logistic regression model of factors measured in the last week prepartum associated with the risk of clinical mastitis in < 30 DIM (median time of occurrence = 2 DIM) in 326 Holstein cattle (batch 2, 23 cases of mastitis, 303 noncases, all untreated with vitamin E injection).

Variable	Level	Parameter estimate	Robust SE	OR ¹	95% CI ²	P
Intercept		-2.222	1.259	...	...	...
Parity group	1	0.001	0.353	1.00	0.50-1.99	0.99
	2	-0.212	0.396	0.81	0.37-1.77	0.59
	≥ 3	Referent	...	...	...	...
Season of calving	Sep-Nov	0.382	0.446	1.46	0.61-3.78	0.39
	Dec-Feb	0.343	0.507	1.41	0.52-3.82	0.50
	Mar-May	0.140	0.534	1.15	0.40-3.29	0.79
	Jun-Aug	Referent	...	...	...	...
Serum retinol concentration	Per 100 ng/mL increase	-0.910	0.270	0.40	0.24-0.68	0.001
Serum cholesterol concentration	Per 1 mmol/L increase	0.636	0.390	1.89	0.88-4.06	0.10

¹Correlation of cows within herd accounted for with generalized estimating equations.

²OR = Odds ratio.

³95 CI = 95% confidence interval.

Table 5. Mixed linear regression model of factors associated with serum α -tocopherol concentration ($\mu\text{g}/\text{mL}$) in peripartum Holstein cattle (785 samples from 310 cows, approximately half of which had retained placenta, batch 1).¹ The covariance parameter estimates were: cow = 0.293 (21%), herd = 0.096 (7%), and residual = 1.018 (73%).

Effect	Level	Estimate	SE	P
Intercept		1.93	0.40	...
Vitamin E injection	Yes	0.47	0.09	<0.0001
Season of calving	Sept–Nov	–0.21	0.13	0.09
	Dec–Feb	0.17	0.12	0.16
	Mar–May	0.06	0.14	0.68
	Jun–Aug	Referent	...	...
Serum cholesterol	1 mmol/L increase	0.35	0.10	0.0004
Serum calcium	1 mmol/L increase	–0.59	0.18	0.001
Serum β -carotene	1 $\mu\text{g}/\text{mL}$ increase	0.48	0.04	<0.0001
Serum retinol	100 ng/mL increase	0.24	0.06	<0.0001
Mastitis < 30 DIM	Yes	0.33	0.14	0.02

¹Repeated measures accounted for with autoregressive correlation structure and intraherd correlation of cows accounted for by inclusion of herd as a random effect in the model.

Table 6. Mixed linear regression model of factors associated with serum α -tocopherol concentration ($\mu\text{g}/\text{mL}$) in peripartum Holstein cattle (1829 samples from 747 cows, none of which had retained placenta, batch 2).¹ The covariance parameter estimates were: cow = 0.025 (3%), herd = 0.032 (3%), and residual = 0.919 (94%).

Effect	Level	Estimate	SE	P
Intercept		1.52	0.25	...
Vitamin E injection	Yes	0.37	0.05	<0.0001
Parity	1	–0.19	0.06	0.002
	2	–0.04	0.06	0.54
	≥ 3	Referent	...	...
Season of calving	Sept–Nov	–0.05	0.06	0.41
	Dec–Feb	–0.15	0.07	0.04
	Mar–May	–0.12	0.07	0.08
	Jun–Aug	Referent	...	...
Serum cholesterol	1 mmol/L increase	0.42	0.06	<0.0001
Serum calcium	1 mmol/L increase	–0.55	0.11	<0.0001
Serum β -carotene	1 $\mu\text{g}/\text{mL}$ increase	0.47	0.04	<0.0001
Serum retinol	100 ng/mL increase	0.15	0.04	<0.0001
Milk fever	Yes	–0.33	0.12	0.004

¹Repeated measures accounted for with autoregressive correlation structure and intraherd correlation of cows accounted for by inclusion of herd as a random effect in the model.

Table 7. Mixed linear regression model of factors associated with serum β -carotene concentration ($\mu\text{g}/\text{mL}$) in peripartum Holstein cattle (785 samples from 310 cows, half of which had retained placenta, batch 1).¹ The covariance parameter estimates were: cow = 0.592 (39%), herd = 0.188 (13%), and residual = 0.726 (48%).

Effect	Level	Estimate	SE	P
Intercept		0.92	0.18	...
Parity	1	–0.13	0.10	0.19
	2	–0.35	0.11	0.002
	≥ 3	Referent	...	...
Season of calving	Sept–Nov	–0.52	0.12	<0.0001
	Dec–Feb	–0.44	0.12	0.0001
	Mar–May	–0.51	0.13	<0.0001
	Jun–Aug	Referent	...	...
Serum NEFA	1 mEq/L increase	–0.02	0.01	0.008
Serum BHB	100 $\mu\text{mol}/\text{L}$ increase	0.14	0.07	0.06
Serum α -tocopherol	1 $\mu\text{g}/\text{mL}$ increase	0.25	0.03	<0.0001
Serum retinol	100 ng/mL increase	0.38	0.05	<0.0001

¹Repeated measures accounted for with autoregressive correlation structure and intraherd correlation of cows accounted for by inclusion of herd as a random effect in the model.

Table 8. Mixed linear regression model of factors associated with serum β -carotene concentration ($\mu\text{g/mL}$) in peripartum Holstein cattle (1829 samples from 747 cows, none of which had retained placenta, batch 2).¹ The covariance parameter estimates were: cow = 0.402 (46%), herd = 0.107 (12%), and residual = 0.364 (42%).

Effect	Level	Estimate	SE	P
Intercept		-0.24	0.13	...
Parity	1	-0.10	0.05	0.03
	2	-0.09	0.04	0.05
	≥ 3	referent	...	...
Season of calving	Sept–Nov	-0.31	0.05	<0.0001
	Dec–Feb	-0.37	0.05	<0.0001
	Mar–May	-0.26	0.05	<0.0001
	Jun–Aug	referent	...	...
Serum cholesterol	1 mmol/L increase	0.30	0.04	<0.0001
Serum glucose	1 mmol/L increase	0.05	0.02	0.005
Serum urea	1 mmol/L increase	0.04	0.01	0.0007
Serum α -tocopherol	1 $\mu\text{g/mL}$ increase	0.15	0.01	<0.0001
Serum retinol	100 ng/mL increase	0.23	0.02	<0.0001

¹Repeated measures accounted for with autoregressive correlation structure and intra-herd correlation of cows accounted for by inclusion of herd as a random effect in the model.

Factors associated with vitamin concentrations were modeled separately for each batch.

Factors Affecting Risk of Retained Placenta and Mastitis

There were no significant associations of any threshold of serum vitamin E concentration with RP, and the significant association of α -tocopherol:cholesterol mass ratio was at a lower cut-point than reported (LeBlanc et al., 2002) or suggested (MSU reference range = α -tocopherol:cholesterol mass ratio 2.5 to 6.0×10^{-3}) elsewhere. However, it should be emphasized that the present values account for the significant effects of several covariates. This makes it difficult to apply these values

directly in the field in the absence of data on these covariates and this underlines the fact that the risk of RP is multifactorial (Kaneene et al., 1997; Kimura et al., 2002). The present results confirm the place of vitamin E as a component of the pathway to RP, but also highlight that there are other important components, one of which may be energy supply for immune function. It is probable that negative energy balance is one element in peripartum immune suppression (Goff and Horst, 1997), and serum NEFA concentration reflects energy balance (Herdt, 2000). This may help to explain why there may not be a consistent threshold of circulating α -tocopherol, assessed in isolation, which neatly and repeatably classifies cows as to risk of RP.

Table 9. Mixed linear regression model of factors associated with serum retinol concentration (ng/mL) in peripartum Holstein cattle (785 samples from 310 cows, half of which had retained placenta, batch 1).¹ The covariance parameter estimates were: cow = 0.07 (<0.1%), herd = 0.494 (15%), and residual = 2743 (85%).

Effect	Level	Estimate	SE	P
Intercept		-78	27	...
Parity	1	9	5	0.10
	2	26	5	<0.0001
	≥ 3	Referent	...	...
Season of calving	Sept–Nov	25	6	<0.0001
	Dec–Feb	2	6	0.78
	Mar–May	2	6	0.82
	Jun–Aug	Referent	...	...
Twins	Yes	-31	10	0.001
RP	Yes	-8	4	0.07
Serum calcium	1 mmol/L increase	59	10	<0.0001
Serum NEFA	1 mEq/L increase	-44	5	<0.0001
Serum cholesterol	1 mmol/L increase	10	5	0.05
Serum urea	1 mmol/L increase	7	2	<0.0001
Serum α -tocopherol	1 $\mu\text{g/mL}$ increase	9	2	<0.0001
Serum β -carotene	1 $\mu\text{g/mL}$ increase	17	2	<0.0001

¹Repeated measures accounted for with autoregressive correlation structure and intraherd correlation of cows accounted for by inclusion of herd as a random effect in the model.

Table 10. Mixed linear regression model of factors associated with serum retinol concentration (ng/mL) in peripartum Holstein cattle (1829 samples from 747 cows, none of which had retained placenta, batch 2).¹

Effect	Level	Estimate	SE	P
Intercept		-9	20	...
Parity	1	14	4	0.0003
	2	17	4	<0.0001
	≥3	Referent	...	...
Season of calving	Sept–Nov	17	4	<0.0001
	Dec–Feb	11	5	0.02
	Mar–May	11	5	0.01
	Jun–Aug	Referent	...	...
Serum calcium	1 mmol/L increase	50	7	<0.0001
Serum NEFA	1 mEq/L increase	-63	4	<0.0001
Serum cholesterol	1 mmol/L increase	14	4	0.0003
Serum urea	1 mmol/L increase	6	1	<0.0001
Serum α -tocopherol	1 μ g/mL increase	6	2	<0.0001
Serum β -carotene	1 μ g/mL increase	22	2	<0.0001

Covariance parameter estimates: cow = 0.14 (<0.1%, herd = 205 (5%), residual = 3654 (95%).

¹Repeated measures accounted for with autoregressive correlation structure and intraherd correlation of cows accounted for by inclusion of herd as a random effect in the model.

In the present analyses, results from blood samples taken between d -6 and 0 relative to calving were associated with risk of RP. This result may be biased by the fact that there were half as many samples from the second week before calving as from the last week before calving, reducing the statistical power in the former time segment. Changes in feed intake and numerous metabolic variables begin 2 wk prepartum, but the largest magnitude of changes generally occur in the 2 wk centered on calving (e.g., NEFA), at calving (e.g., calcium) or in the first 2 wk postpartum (e.g., BHBA). In the case of vitamin E, circulating concentrations generally parallel the drop and rebound in dry matter intake in the transition period (Weiss et al., 1994). With respect to the occurrence of RP or mastitis, the truly important sites of action of vitamins E and A may be within uterine and mammary neutrophils and epithelial cells, respectively. Questions remain about when, how, and to what degree dietary or parenteral supplementation of vitamin E results in increased intra-neutrophil concentration of α -tocopherol, and the duration of such an effect (Weiss et al., 1992; Weiss et al., 1994). Preliminary data (LeBlanc and Bankert, 2002, unpublished) suggest that following injection of 3000 IU of RRR-tocopheryl acetate i.m. or s.c., the concentration of α -tocopherol within circulating neutrophils closely mirrors plasma concentrations, which peak 2 to 3 d after injection, and decline by 5 to 7 d after injection.

Interestingly, the mastitis model reduced to one significant main effect, retinol, with a tendency for an association of cholesterol with risk of mastitis. Increasing serum retinol concentration was associated with decreased risk of clinical mastitis (60% relative reduction in risk per 100 ng/mL increase in retinol, within the range of values observed; mean $\pm$ SD = 191 $\pm$ 76

ng/mL). The confidence interval around the estimated effect of increasing serum cholesterol lies mostly above 1, suggesting a positive association. Serum cholesterol concentrations generally decline from 2 wk before calving until 1 to 2 wk after calving (Kaneene et al., 1997). Cholesterol is a component of lipoproteins and is considered a marker in peripartum cows for hepatic export of lipid. Therefore, higher serum cholesterol concentration may reflect better hepatic function in exporting lipoproteins in the face of increased hepatic influx of mobilized fatty acids. The observed association is consistent with the hypothesis that liver function during peripartum negative energy balance is a component of the risk of early lactation mastitis.

Unlike the RP model, possible threshold values of retinol for risk of mastitis were not calculated because the absolute values of retinol in the dataset were suspect. However, this problem should not affect the significance and relative magnitude of the effects in the model, only their translation into absolute values.

Although the possible protective effect of dietary vitamin E supplementation on mastitis is well publicized, in the present analysis there was no association between serum α -tocopherol concentration in the last week prepartum, and the risk of mastitis at or soon after calving. In many reports on vitamin E in transition dairy cows, both controlled experiments investigating supplemental vitamin E and observational studies, covariate data were not collected on metabolic measures of energy balance (e.g., NEFA) or concentrations of other potentially important nutrients. In the present investigation, the lack of association between serum vitamin E and mastitis is likely attributable in part to most cows receiving some dietary supplemental vitamin E, and to simultaneous consideration of other metabolic factors that may

contribute to the occurrence of mastitis. Although there are numerous studies demonstrating a benefit of dietary supplementation with 1000 IU vitamin E/d relative to lower levels, there is some evidence that for reduction of risk of mastitis in particular, dietary supplementation of 2000 to 4000 IU/d may be appropriate (Weiss et al., 1997; Baldi et al., 2000). Hogan et al., 1993 showed that the optimum plasma concentration of α -tocopherol for mammary cell immune function was 3.5 to 4.0 $\mu\text{g/mL}$. Very few cows in the present study population maintained this threshold, even if the data were adjusted for the batch effect.

Notably, despite the large sample sizes for a study with a broad profile of weekly metabolic data, both the RP and mastitis models reduced to only two metabolic variables. Also notable was the lack of association of β -carotene concentration with either disease. Although the present study offers no mechanistic data, the results are not consistent with reports of enhanced immune function attributed to β -carotene (Michal et al., 1994). Weiss et al. (1994) found essentially no β -carotene within neutrophils at calving. It is worth underlining that RP and mastitis are multifactorial diseases, with energy balance, liver function, and fat-soluble vitamin status as key components of immune function and therefore of resistance to these conditions.

Factors Associated with Peripartum Serum Vitamin Concentrations

With respect to determinants of peripartum serum concentrations of α -tocopherol, β -carotene, and retinol, major factors such as DMI and the level of dietary supplementation were not measured in this study. Dry matter intake and diet composition similarly have significant influence on metabolites such as NEFA and cholesterol. However, in a large, multi-herd field study with the cow as the unit of interest, it was not feasible to collect detailed information on diets.

Note that the models for each batch are independent; therefore the absolute values of vitamin concentrations are not directly comparable between batches. However, the magnitudes of effects on vitamin concentrations are comparable between models. Because the batch 2 dataset was larger, there was inherently greater statistical power to detect effects, and this contributed to differences in the variables in the final models.

Although serum α -tocopherol concentration in the last week prepartum was associated with the probability of RP (Table 2), conversely, when all other significant covariates were included the occurrence of RP did not significantly affect the average serum α -tocopherol concentration from 1 wk before expected calving through the first week postpartum (Table 5). There was good

agreement between the α -tocopherol models. Peripartum serum α -tocopherol concentrations were highest in the summer, most likely associated with consumption of pasture or recently harvested forages. As expected, serum cholesterol concentration was positively associated with serum α -tocopherol concentration. Cholesterol has been shown to be highly correlated with circulating lipoprotein concentration, and high-density lipoproteins are the main carrier of α -tocopherol in circulation (Herdt and Smith, 1996). Serum concentrations of β -carotene and retinol were positively associated with serum α -tocopherol concentrations. Although the amount of dietary vitamin supplementation was unknown, if dietary vitamin E supplementation were aggressive (e.g., ≥ 1000 IU/d) then vitamin A may also be supplemented above traditional levels.

There was generally good agreement between the two β -carotene models on the direction and magnitude of the effects common to both models. There was greater variability in the final metabolic variables than in the models for α -tocopherol and retinol. However, the fact that inclusion of different metabolic variables did not substantially change the estimates of the effects of parity, season, α -tocopherol, and retinol suggests that the metabolic variables are largely independent of the former effects. The 2 models retained different metabolic variables, but each set contained measures of energy balance. It is possible that the associations with measures of energy balance could be confounded by increased release of stored fat-soluble vitamins as adipose tissue is catabolized. Although statistically significant, given the expected ranges of all of these metabolic variables, their effects on circulating β -carotene concentration are small.

Again, there was agreement between the 2 models for retinol. In both models, all associations between retinol and metabolic and other vitamin measures were in the same direction and of similar magnitude. However, practically, the influence of changes in these variables on retinol concentrations may not be large. For example, the typical peripartum range of calcium concentration is 1.9 to 2.4 mmol/L, therefore the model predicted that movement from the bottom to the top of that range only increased retinol by 25 ng/mL. Most peripartum cows will have between 2.0 and 2.3 mmol/L of total calcium. Similarly, the expected range of postpartum NEFA concentration is 0.5 to 1.0 mEq/L for healthy cows; therefore, an increase from the bottom to the top of that range would be expected to reduce retinol by only 30 ng/mL.

As expected, there was a positive association between circulating concentrations of β -carotene and retinol. There was also a significant but smaller per unit association between serum concentrations of α -tocopherol

and retinol. Again, however, magnitudes of changes in these measures that are likely to be seen in practice would not be expected to produce changes in retinol concentrations that would have substantial health effects.

CONCLUSIONS

The present results highlight the nondietary factors that influence the circulating concentrations of vitamins A and E. The results point to associated metabolic factors that may merit consideration in investigation of unexpected responses to dietary vitamin supplementation in certain cases.

The models of factors affecting risk of RP and mastitis highlight the multifactorial nature of peripartum immune function that underlies both conditions. Although the present results confirm the protective roles of α -tocopherol and retinol in the pathways to RP and peripartum mastitis, respectively, there may not be simple thresholds of these to be achieved to prevent the disease conditions. Rather, they are components of a larger causal web for these biologically and economically important diseases.

Future research should consider greater elucidation of the timing, magnitude, and mechanisms of movement of vitamins A and E into effector tissues for health in peripartum dairy cows, in the context of the key associated effects of energy metabolism in these animals.

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