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Changes in Vitamin C Concentrations in Plasma and Milk from Dairy Cows After an Intramammary Infusion of *Escherichia coli**

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ABSTRACT

Plasma and milk concentrations of ascorbic acid and dehydro-L-ascorbic acid (DHAA) were measured before and after 21 Holstein cows (approximately 26 DIM) were given an intramammary infusion of *Escherichia coli*. Blood, milk from the unchallenged quarters, and milk from the challenged gland were sampled immediately before challenge (d 0) and 24 h and 7 d postchallenge. Plasma vitamin C (ascorbic acid + DHAA) concentrations decreased 39%, and concentrations of vitamin C and ascorbic acid in milk from the challenged quarter decreased 52 and 62%, respectively, in samples taken 24 h postchallenge. No change was observed in vitamin C concentrations in milk from unchallenged quarters. The concentration of DHAA in milk from challenged quarters increased 67% 24 h postchallenge. The duration of clinical mastitis, peak body temperature, number of colony-forming units of *E. coli* isolated from the infected gland, and loss in milk yield were associated with a change in concentration of vitamin C in milk from the challenged quarter. Increased severity of clinical signs was associated with large decreases in concentration of vitamin C in milk from the challenged quarter. Similar, but statistically weaker, relationships were observed for changes in plasma vitamin C concentrations.
(**Key words:** ascorbic acid, vitamin C, mastitis)

Abbreviation key: DHAA = dehydro-L-ascorbic acid.

INTRODUCTION

Neutrophils are a primary host defense mechanism against IMI, and responsiveness of neutrophils is related to the incidence and severity of mastitis in dairy cows (Craven and Williams, 1985). Neutrophil-killing ability is increased (Hogan et al., 1992), and incidence and severity of mastitis (Smith et al., 1984) are reduced when cows are supplemented with vitamin E and selenium, two antioxidant nutrients. Ascorbic acid (vitamin C) is the most abundant and probably most important water-soluble antioxidant in mammals (Sauberlich, 1994) and is found in most, if not all, tissues and biological fluids (Schorah, 1992). The concentration of L-ascorbic acid in unstimulated human neutrophils is extremely high and increases approximately 10-fold when the neutrophil is stimulated (Washko et al., 1995). Upon infection and stimulation of neutrophils, the demand by those cells for ascorbic acid may draw down body pools. The high concentration of ascorbic acid may be needed to protect the cells from the oxidants produced from the respiratory burst.

Vitamin C is not considered to be an essential dietary nutrient for healthy dairy cows. However, the high concentrations of ascorbic acid found in human neutrophils and the relationships between neutrophil function, mastitis, and essential dietary antioxidants (i.e., vitamin E and Se) led us to postulate that vitamin C status of dairy cows might be compromised during an IMI. The objective of this experiment was to determine whether vitamin C status of dairy cows was affected by acute coliform mastitis.

MATERIALS AND METHODS

Twenty-one lactating Holstein cows were divided into 8 groups (3 groups were primiparious cows) of 2 or 3 cows based on calving date. Cows were housed in a tie-stall barn and fed a common diet that met or exceeded NRC requirements (NRC, 2001; Table 1). Cows were milked twice daily and fed once daily. Individual daily milk yield and DMI were recorded during the experiment. At approximately 26 DIM (ranged from 14 to 39 DIM), all cows within a group received an intramammary infusion of *Escherichia coli* 727, originally isolated from a naturally occurring IMI, into either the right or left front mammary quarter. Challenge inoculum was prepared by inoculation of a frozen stock culture of

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Table 1. Diet fed to cows from parturition until 14 d after intramammary challenge with *Escherichia coli*.

Item	% of DM
Corn silage	16.9
Alfalfa silage	29.4
Alfalfa hay	9.1
Corn grain, ground	27.7
Soybean meal, 44% CP	5.6
Roasted whole soybeans	5.6
Soypass	1.5
Soybean hulls	1.9
Mineral mix[1]	1.5
Vitamin mix[2]	0.8
Nutrients	
CP	16.7
NDF	32.5
Ca	0.62
P	0.32
Cu, mg/kg	20
Zn, mg/kg	52

[1]Mineral mix contained 34.07% sodium bicarbonate, 25.84% sodium chloride, 12.14% limestone, 8.53% magnesium oxide, 5.65% dicalcium phosphate, 4.17% monosodium phosphate, 8.70% sodium selenate premix (200 mg/kg), 0.24% copper sulfate, and 0.66% Zinpro 100 (Zinpro Corp., Eden Prairie, MN).

[2]Vitamin premix contained 470,000 IU of vitamin A, 139,000 IU of vitamin D, and 2400 IU of vitamin E/kg.

E. coli 727, as described in Hogan et al. (1995). The geometric mean of the colony-forming units for challenge inoculum was 72 (range: 65 to 77 cfu) suspended in 1 ml of PBS. Infusions were given 3 h after morning milking, and only uninfected quarters were infused.

Quarter foremilk samples also were collected 7, 5, and 3 d prior to bacterial challenge; immediately prior to challenge; 3, 6, 9, 12, 15, 18, 21, and 24 h and on d 2, 3, 4, 7, and 14 postchallenge. Sample collection and microbiological procedures were as previously described (Todhunter et al., 1991). The number of colony-forming units per milliliter was determined in quarter foremilk samples during the postchallenge period. Colony-forming units were determined by 10-fold dilutions of sample in PBS. The initial inoculates were duplicate 1-mL pour plates of undiluted milk in McConkey agar. Dilutions were plated on the surface of McConkey agar plates. All dilutions were in duplicate. Data were expressed as $\log_{10}$ cfu/mL of milk. An IMI was diagnosed when bacteria were isolated from 2 consecutive samples. The duration of IMI was the hours between first and last isolation of bacteria from a quarter. Clinical status of all quarters was recorded at the time quarter foremilk samples were obtained. Clinical mastitis was defined when abnormal milk, a swollen quarter, or systemic signs of infection were observed. Rectal temperatures were measured immediately prior to challenge and at each time that quarter foremilk samples were collected postchallenge.

Immediately before challenge, a blood sample was taken from the tail vein into heparin-containing tubes. Milk from the quarter that was to be challenged and a composite sample from the other three quarters also was sampled (d 0 samples). This sampling protocol was repeated at 24 h and 7 d postchallenge. All samples were placed on ice and transported to the laboratory (approximately 1 h). Upon arrival in the laboratory, blood was immediately centrifuged to obtain plasma. A subsample of milk from all challenged quarter was immediately extracted and assayed for ascorbic acid (Timmons et al., 2001). The plasma, milk from the unchallenged quarters, and a subsample of milk from the challenged quarter (all cows) were acidified and reduced (using dithiothreitol) within 2 h of obtaining the sample (Timmons et al., 2001). This process converts dehydro-L-ascorbic acid (**DHAA**) into ascorbic acid and prevents oxidation of ascorbic acid. The concentration of ascorbic acid in these samples were assayed within 3 d (samples stored at 4 C in the dark) using HPLC (Timmons et al., 2001). The resulting value represents total vitamin C (i.e., ascorbic acid plus DHAA). A subsample of plasma from 4 groups (n = 12) was also immediately extracted for ascorbic acid analysis. The concentration of DHAA in milk from the challenged quarter (all cows) and plasma (12 cows) was calculated as total vitamin C minus ascorbic acid. Ascorbic acid in the absence of dithiothreitol is labile and must be assayed soon after samples are collected. Logistics prevented us from assaying all plasma samples for ascorbic acid and DHAA.

The effects of time postchallenge on concentrations of ascorbic acid, DHAA, and vitamin C were analyzed statistically using Proc MIXED (SAS Inst., Inc., Cary, NC). The model included group (random variable, 7 df), time (repeated fixed effect, 2 df), and error (53 df). Compound symmetry was the covariance structure used. The time effect was partitioned into 2 contrasts: d 0 vs. 24 h, and d 0 vs. d 7. The same model was used for plasma ascorbic acid and DHAA except that that group had only 4 df and error had 30 df. Regression analysis (Proc REG; SAS Inst., Inc.) was used to quantify relationships among variables of interest.

RESULTS AND DISCUSSION

Maximal body temperature, peak *E. coli* bacterial counts, and depression in milk yield occurred by 1 d postchallenge (data not shown); by 7 d postchallenge, all values returned to prechallenge levels. Geometric mean duration of clinical signs was 27 h. Milk yield during the first 24 h postchallenge decreased 36% compared with the preceding day, but milk yield by 7 d postchallenge were similar to prechallenge values (Ta-

Table 2. Effect of intramammary infusion of *Escherichia coli* on concentrations of ascorbic acid (AsA), dehydro-L-ascorbic acid (DHAA), and vitamin C (AsA + DHAA) in milk and plasma. Values are from 21 cows except were noted.[1]

Item	0 d	24 h PC	7 d PC	SE
Milk yield, kg/d[a]	38.8	24.6	36.9	1.83
DMI, kg/d[b]	18.1	17.1	20.7	0.9
Plasma				
Vitamin C, mg/L (n = 21)[2 ac]	4.72	2.86	4.39	0.23
Vitamin C, mg/L (n = 12)[3 a]	4.78	3.13	4.83	0.29
AsA, μmol/L (n = 12)[3 a]	29.9	17.3	29.7	2.0
DHAA, μmol/L (n = 12)[3]	1.3	2.1	0.5	0.5
DHAA, mol/mol of vitamin C (n = 12)[3 a]	0.044	0.106	0.017	0.024
Milk-challenged quarter				
Vitamin C, mg/L[ab]	22.87	10.96	20.00	1.20
AsA, μmol/L[ac]	131.4	49.3	118.6	8.6
DHAA, μmol/L[ac]	10.6	17.7	5.7	2.8
DHAA, mol/mol of vitamin C[a]	0.082	0.306	0.045	0.035
Milk-unchallenged quarters				
Vitamin C, mg/L[c]	22.50	23.61	20.82	1.32

[a]Day 0 differs from 24 h ($P < 0.05$).

[b]Day 0 differs from d 7 ($P < 0.05$).

[c]Day 0 differs from d 7 ($P < 0.10$).

[1]The 0-d sample was taken before challenge; other samples were taken at 24 h and 7 d postchallenge (PC).

[2]Total vitamin C measured in all cows (n = 21).

[3]Plasma concentrations of vitamin C, AsA, and DHAA were measured in a subset of 12 of the 21 cows in the experiment.

ble 2). Dry matter intake was not reduced the day after challenge and was higher on d 7 than on d 0, reflecting the normal increase in DMI during early lactation. These responses were similar to those from previous challenge experiments using the same strain of *E. coli* (Todhunter et al., 1991; Barrett et al., 1997).

Change in Plasma and Milk Vitamin C

Mean concentrations of plasma vitamin C for all cows (n = 21) and the subset of 12 cows in which plasma vitamin C was partitioned into ascorbic acid and DHAA were similar (Table 2); therefore, ascorbic acid and DHAA values obtained from the subset are assumed to represent all cows. Plasma concentrations of vitamin C were similar on d 0 (prechallenge) and 7 d postchallenge (Table 2). The concentrations of vitamin C in plasma collected on those 2 time points were higher than previously reported (Hidiroglou et al., 1995; Hidiroglou, 1999; Santos et al., 2001; Weiss, 2001) plasma concentrations in lactating dairy cows (range: 2.5 to 3.8 mg/L). The reasons for the difference are not clear. Experiments used different analytical techniques to measure vitamin C and diets and milk production varied among experiments (cows in the previous experiments produced 8 to 20 kg/d less milk and were generally fed high forage diets). On d 0 and 7, DHAA comprised less than 5% of total vitamin C. In a previous experiment, DHAA contributed about 10% to total plasma vitamin C (Weiss, 2001). Plasma concentrations of vitamin C decreased 39% ($P < 0.01$) by 24 h postchallenge (Table 2). Essentially all of the decrease in vitamin C was caused by a decrease in ascorbic acid concentrations, thereby increasing the proportion of vitamin C contributed by DHAA in the 24 h samples.

The concentrations of vitamin C in d-0 milk from the quarter used for the challenge and milk from the unchallenged quarters were essentially the same and averaged 22.7 mg/L (Table 2). Hartman and Dryden (1978) reported a mean concentration of 20.9 mg/L for fresh milk from a summary of literature values. In d-0 milk, DHAA made up about 8% of the total vitamin C, which was similar to that reported previously (Weiss, 2001). Barrefors et al. (1995) reported that about 70% of the vitamin C in milk was DHAA but that high value could be an artifact of sample storage because ascorbic acid is converted to DHAA over time.

The vitamin C concentration in milk from the unchallenged quarters changed only slightly over the experiment (Table 2) and no change was observed from d 0 to 24 h postchallenge. However, concentrations of vitamin C and ascorbic acid in milk from the challenged quarter collected 24 h postchallenge were markedly lower ($P < 0.01$) than prechallenge concentrations. In the challenged quarter, vitamin C concentrations decreased 52% and ascorbic acid concentrations decreased 62% from d 0 to 24 h postchallenge. Conversely, the concentration of DHAA in the challenged quarters was

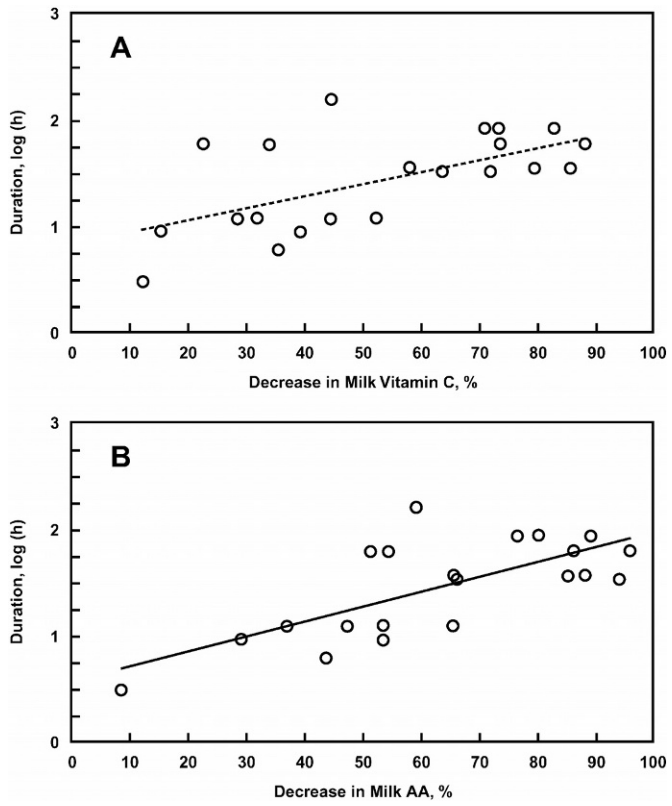

Figure 1. Relationships between duration of clinical mastitis and decreases in vitamin C (A) or ascorbic acid (B) concentrations in milk from a quarter challenged with *Escherichia coli*. Decrease was calculated as the concentration at d 0 (prechallenge) minus concentration 24 h postchallenge divided by d 0 times 100. Dashed line = 0.83 (± 0.20) + 0.012 (± 0.003)X; $P < 0.01$; root mean square error (RMSE) = 0.37; $r^2 = 0.37$. Solid line = 0.56 (± 0.21) + 0.014 (± 0.003)X; $P < 0.01$; RMSE = 0.33; $r^2 = 0.50$.

almost twice as high 24 h postchallenge than prechallenge concentrations, and the proportion of vitamin C that was DHAA increased ($P < 0.01$) from about 8 to 31%. At 7 d postchallenge, vitamin C concentrations in milk from the challenged quarter was about 12% lower ($P < 0.05$) than prechallenge concentrations. Concentrations of ascorbic acid and DHAA tended to be lower ($P < 0.10$) on d 7 than on d 0.

The decrease in plasma vitamin C concentrations during the first 24 h postchallenge was caused by decreased synthesis of ascorbic acid, increased uptake of ascorbic acids by cells, or increased oxidation of ascorbic acid in the plasma. Ascorbic acid is synthesized from glucose, and glucose synthesis does not appear to be reduced during mastitic episodes when DMI is not reduced (Shuster et al., 1991) and DMI was not reduced in our study. Some of the oxidants produced by the respiratory burst of neutrophils and other immune cells could enter the circulation and oxidize plasma ascorbic acid, thereby reducing its concentration. If this were occurring extensively, concentrations of DHAA would be expected to increase because DHAA is the initial product produced when ascorbic acid is oxidized. The concentration of DHAA in plasma increased about 60% between d 0 and 24 h postchallenge, but this was not significant ($P > 0.16$). The lack of a statistical effect could reflect a true lack of response or could be caused by insufficient observations (plasma DHAA was measured in only 12 cows). However, the increased proportion of plasma vitamin C as DHAA suggests that increased oxidation of ascorbic acid in the plasma occurred postchallenge. A third reason for decreased plasma vitamin C is increased uptake by cells. Neutrophils, when stimulated, take up large quantities of ascorbic acid (Wang et al., 1997). After the mammary gland challenge, blood neutrophils would become stimulated and their uptake of ascorbic acid would draw down plasma ascorbic acid concentrations.

Changes in vitamin C concentrations in milk from the challenged gland reflect local rather than systemic events. The decrease in vitamin C and ascorbic acid in milk from the challenged quarter is probably not directly caused by low plasma concentrations because concentrations of vitamin C in the unchallenged quarters were not affected by challenge, and in a study with healthy cows, the secretion rate of vitamin C into milk was not limited until plasma concentrations of ascorbic acid were less than approximately 1.6 mg/L (Weiss, 2001). Rather, the data suggest that the decrease in milk ascorbic acid and vitamin C in the infected quarter

Table 3. Significant ($P < 0.05$) correlations (r) between decreases in concentrations of vitamin C (VC) and ascorbic acid (AsA) in blood and milk from the challenged gland and clinical signs (N = 21 except where noted).[1]

Item	Milk VC	Milk AsA	Plasma VC
Duration clinical mastitis, log (h)	0.61	0.71	NS
Duration of IMI, log (h)	NS	0.44	0.42
Peak *Escherichia coli* counts, $\log_{10}$ cfu/ml	0.61	0.54	NS
Peak body temperature, °C	0.63	0.58	0.53
Decrease in milk yield, %	0.86	0.78	0.63

[1]Decrease was calculated as follows: [100 × concentration at d 0 – concentration 24 h postchallenge]/d 0.

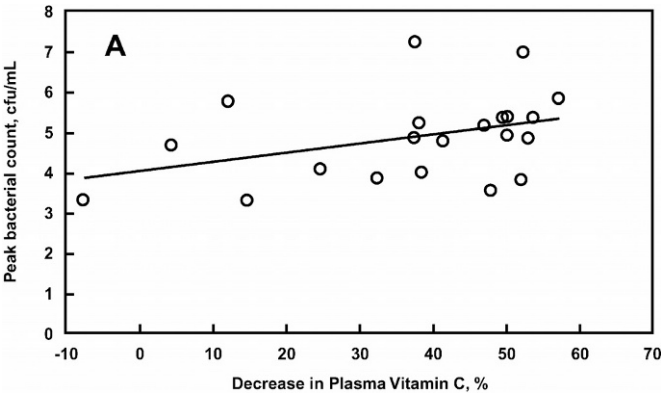

Figure 2. Relationships between peak *Escherichia coli* counts in the challenged quarter and decrease in vitamin C concentrations in milk from the challenged quarter. Decrease was calculated as d 0 (prechallenge) concentration minus concentration at 24 h postchallenge divided by d 0 times 100. Solid line = 3.44 (± 0.47) + 0.027 (± 0.008)X; $P < 0.01$; root mean square error = 0.87; $r^2 = 0.37$.

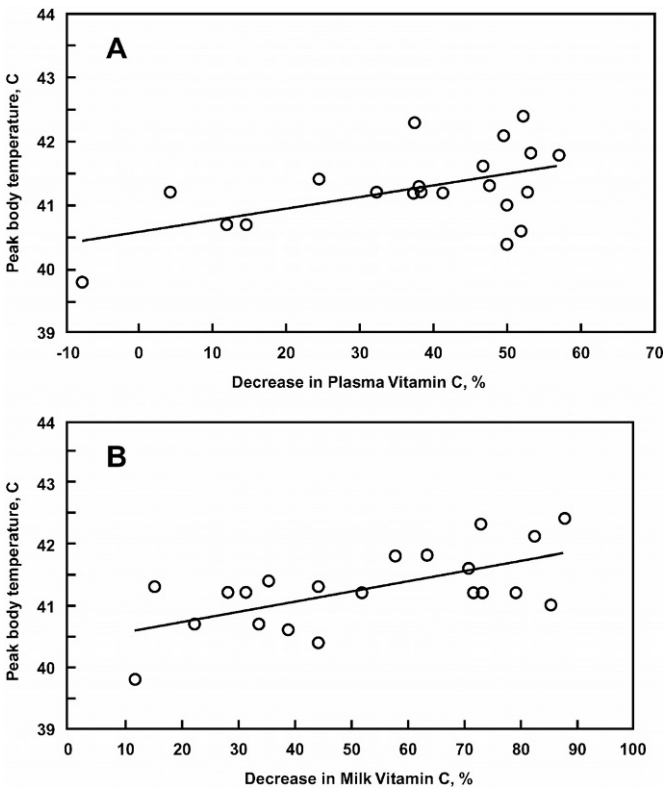

Figure 3. Relationships between peak body temperature and decrease in vitamin C concentrations in (A) plasma or (B) milk from the quarter challenged with *Escherichia coli*. Decrease was calculated as d 0 (prechallenge) concentration minus concentration at 24 h post challenge divided by d 0 times 100. Dashed line = 40.6 (± 0.3) + 0.018 (± 0.007)X; $P < 0.02$; root mean square error (RMSE) = 0.54; $r^2 = 0.28$. Solid line = 40.4 (± 0.3) + 0.016 (± 0.005)X; $P < 0.01$; RMSE = 0.50; $r^2 = 0.40$.

was caused mostly by increased utilization. The respiratory burst of neutrophils during phagocytosis produces numerous free radicals and reactive oxygen compounds (Laurent et al., 1991). These compounds are found within the neutrophil and in the extracellular environment (i.e., milk in the ductile tissues of the infected gland). Ascorbic acid in the milk would be oxidized by the reactive oxygen compounds initially producing DHAA (as evidenced by the approximately 70% increase in DHAA in milk from the challenged gland; Table 2). The magnitude of change in milk DHAA, however, was not significantly correlated with any clinical response. Ascorbic acid within the neutrophil would also be oxidized by reactive oxygen metabolites found within the cell. Intracellular and extracellular oxidation of ascorbic acid would reduce the concentration of ascorbic acid in milk from the challenged quarter and increase concentrations of DHAA, as we observed in this study. The decrease in total vitamin C (i.e., ascorbic acid + DHAA) means that a portion of the DHAA produced was oxidized irreversibly to 2,3-diketogluonic acid.

Relationships with Clinical Signs

Greater decreases (24 h vs. d 0) in concentrations of vitamin C in milk from the challenged quarter were associated with increased duration of clinical mastitis (Figure 1; Table 3). Similar relationships were found for peak body temperature (Figure 2), peak number of colony-forming units of *E. coli* isolated from the challenged gland (Figure 3), and change in milk yield (Figure 4). No consistent differences in the statistical relationships between clinical signs and the change in vitamin C or ascorbic acid concentrations in milk from the challenged gland were observed (Table 3). The decrease in plasma vitamin C concentrations was also associated with peak body temperature (Figure 3) and change in milk yield (Figure 4). Statistical associations between clinical signs and change in plasma vitamin C were weaker than relationships with change in vitamin C concentration from the challenged gland (Table 3).

Whether increased severity of mastitis caused a greater decrease in plasma and milk vitamin C or whether severity increased because inadequate vitamin C was available cannot be determined from these data. Intravenous infusions of 25 g of ascorbic acid 3 and 5 h after an intramammary gland challenge with endotoxin did not affect febrile response in dairy cows but did help improve recovery of milk production postchallenge (Chaiyotwittayakun et al., 2002). Santos et al. (2001) found no correlation between plasma ascorbic acid concentrations and SCC; however, mean SCC in that study was <120,000 cells/mL, indicating low incidence of clini-

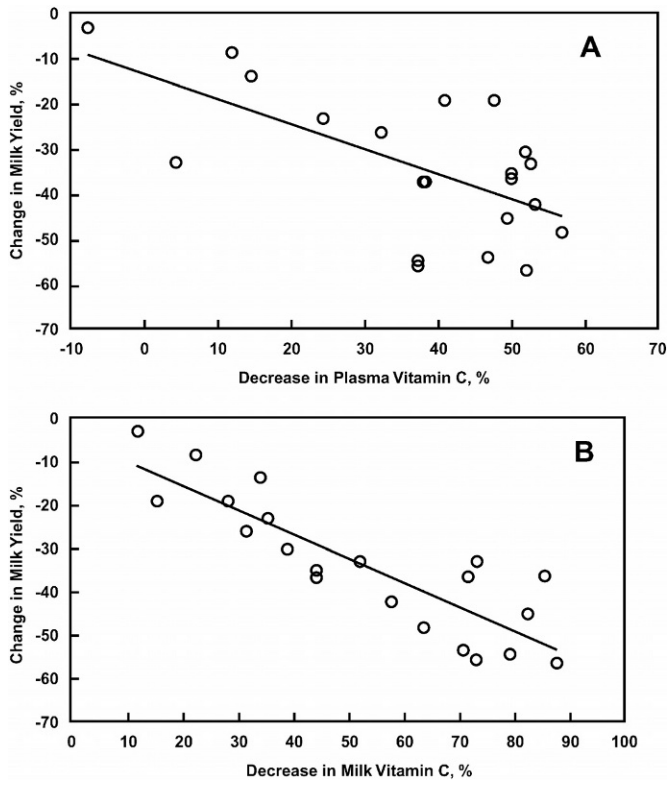

Figure 4. Relationships between change in total milk yield and decrease in vitamin C concentrations in (A) plasma or (B) milk from the quarter challenged with *Escherichia coli*. Percent change = 100 × [(yield during 24 h post challenge) − (milk yield on d 0 before challenge)]/d 0. Decrease = absolute value of change. Dashed line = −13.4 (± 6.4) − 0.55 (± 0.15)X; $P < 0.01$; root mean square error (RMSE) = 12.3; $r^2 = 0.40$). Solid line = -4.5 (± 4.3) − 0.56 (± 0.08)X; $P < 0.01$; RMSE = 8.1; $r^2 = 0.74$.

cal mastitis. In our study, concentrations of vitamin C in plasma or milk on d 0 were not correlated with subsequent clinical responses following challenge (data not shown). Clinical studies are needed to determine whether vitamin C has therapeutic or prophylactic value in reducing the incidence and severity of coliform mastitis.

CONCLUSIONS

An intramammary infusion of *E. coli* caused large decreases in concentrations of vitamin C and ascorbic acid in plasma and milk from challenged quarter indicating that an inflammatory response increases oxidation of ascorbic acid. Larger decreases in plasma and milk vitamin C and ascorbic acid occurred as severity of mastitis increased.

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