Effects of vitamin E supplementation on and the association of body condition score with changes in peroxidative biomarkers and antioxidants around calving in dairy heifers


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ABSTRACT

The objective of this study was to investigate the effect of vitamin E supplementation on oxidative status in blood, liver, milk, and ovarian follicular fluid in periparturient heifers. Vitamin E supplementation started 8 wk before calving and continued until 8 wk postpartum. Grass silage was the main forage fed during the experiment. In addition, supplemented heifers (n = 9) received 3,000 IU of vitamin E daily on a carrier food; control heifers (n = 9) consumed only the carrier food. Blood samples and liver biopsies were taken frequently throughout the study and ovarian follicular fluid was sampled at 8 wk postpartum. Body condition score was scored weekly and milk yield was measured daily. A marker of oxidative damage, determinable reactive oxygen metabolites (d-ROM), and a set of antioxidants were measured in blood, liver, milk, and ovarian follicular fluid. Control heifers had a low vitamin E status, and selenium status was marginal in control and supplemented heifers. Vitamin E supplementation increased vitamin E concentrations in blood, liver, and ovarian follicular fluid and increased triacylglycerol in liver. Serum d-ROM were not reduced by vitamin E supplementation. Superoxide dismutase and glutathione peroxidase activity in red blood cells and liver and glutathione peroxidase activity in ovarian follicular fluid were not affected by vitamin E supplementation and they were not increased around calving. Protein thiol groups and ratio of reduced glutathione to oxidized glutathione were also not increased around calving. These results suggest that heifers around calving experience a low level of oxidative processes. This might be caused by lower than expected milk production attributed to a low forage intake. Serum d-ROM were negatively correlated with protein thiol groups and positively correlated with the activity of glutathione peroxidase in red blood cells, oxidized glutathione, and the ratio of reduced glutathione and oxidized glutathione in serum. The lack of treatment effects allowed estimation of the effects of body condition 4 wk before calving and the loss of body condition on markers of lipid peroxidation and antioxidants. A trend that a body condition of ≥3 might result in more oxidative damage measured by serum d-ROM was observed, but fatter heifers had a significantly higher ratio of reduced glutathione to oxidized glutathione.

Key words: body condition score, dairy cow, oxidative status, vitamin E

INTRODUCTION

The transition from gestation to lactation in dairy cows is accompanied by an increased risk for several metabolic and infectious disorders that may depress milk production (Drackley, 1999; Østergaard and Gröhn, 1999). The periparturient period involves major metabolic changes associated with the initiation of and rapid increase in milk production. The abruptly increased metabolic rate concomitantly results in a greater release of reactive oxygen species (ROS; Halliwell and Gutteridge, 2007). An imbalance between metabolic entry and disposal of ROS was suggested to contribute to periparturient disorders in cows (Miller et al., 1993; Sordillo and Aitken, 2009). At calving, the antioxidant defense system may not yet have adapted to the increased ROS supply. One of the primary oxidants is the superoxide anion, which is removed by the enzyme superoxide dismutase (SOD). Although the activity of SOD increases after calving in dairy cows (Stefanon et al., 2005; Gaál et al., 2006; Colitti and Stefanon, 2006), several recent studies have shown that the antioxidative capacity in periparturient dairy cows is insufficient to counteract the increase in ROS supply.
Lipid accumulation is known to impair the quality of the ovonic development (Olson and Seidel, 2000). Excessive sized oocytes with vitamin E improved bovine embryogenesis (Tatemoto et al., 2004). Accordingly, culturing fertilized follicular fluid positively affects oocyte quality in pigs (et al., 2003), and the antioxidant capacity in ovarian damage can be detrimental to bovine embryos (Feugang et al., 2009), In addition, oxidative stress may improve overall health and fertility in dairy cows (Spears, 2000). Supplementing vitamin E may improve health during the periparturient period when plasma α-tocopherol is lowest (Goff and Stabel, 1990). The incidence of mastitis, for example, was reduced after supplementation of vitamin E during the dry period (Weiss et al., 1997; Moyo et al., 2004), and the numbers of services per conception and days to conception were lower in cows supplemented with 2,000 IU of vitamin E/d than in those receiving 1,000 IU/d (Stowe et al., 1988; Baldi et al., 2000). Although such beneficial effects of vitamin E on health are often attributed to reduced oxidative damage (Aitken et al., 2009), markers for oxidative status have only occasionally been measured in dairy cows supplemented with vitamin E.

Markers in plasma and erythrocytes may provide good estimates of the balance between oxidants and antioxidants on whole-body level (et al., 1994; Bernabucci et al., 2005). Specific tissues may, however, differ in oxidative status, which cannot be derived from plasma or erythrocyte values but could involve oxidative damage at those specific sites. In humans, urinary concentrations of thiobarbituric acid reactive substances (TBARS) increased following consumption of a diet high in polyunsaturated fatty acids, but these changes were not reflected in concentrations of TBARS and dienes in serum (Jenkinson et al., 1999). In cattle, effects of vitamin E supplementation on malondialdehyde (MDA), a degradation product of lipid peroxidation, differed between blood, liver, and mammary gland (Bouwstra et al., 2008), and expression of genes coding for pro- and antioxidant enzymes was increased in the mammary glands of periparturient dairy cows (Aitken et al., 2009). In addition, oxidative damage can be detrimental to bovine embryos (Feugang et al., 2003), and the antioxidant capacity in ovarian follicular fluid positively affects oocyte quality in pigs (Tatemoto et al., 2004). Accordingly, culturing fertilized oocytes with vitamin E improved bovine embryonic development (Olson and Seidel, 2000). Excessive lipid accumulation is known to impair the quality of the embryos by oxidative stress (Leroy et al., 2005). Mechanisms by which vitamin E affects health in periparturient dairy cows may thus be identified by measuring responses of oxidative status in various tissues.

Therefore, the aim of this study was to quantify the effects of dietary vitamin E supplementation on one peroxidation marker, determinable reactive oxygen metabolites (d-ROM), and on a set of antioxidants in blood, liver, and ovarian follicular fluid in periparturient heifers. It was hypothesized that supplementing 3,000 IU of vitamin E per day from 8 wk before until 8 wk after calving would increase antioxidant and decrease oxidant concentrations in heifers, especially during the week around calving. Moreover, we expected that this effect would be more pronounced in heifers with a higher BCS, because they may experience more oxidative stress during the transition period (Bernabucci et al., 2005; O’Boyle et al., 2006).

**MATERIALS AND METHODS**

**Animals, Housing, and Feeding**

Clinically healthy Holstein heifers (n = 18) with anticipated calving dates in a window of 2 wk were purchased and housed in tie-stall barns at the Department of Farm Health at Utrecht University (the Netherlands). Heifers were matched for BCS and liver iron status at 9 wk before calving, because iron may act as a pro-oxidant (Symons and Gutteridge, 1998), and then assigned to the vitamin E–supplemented group (n = 9) or the control group (n = 9). Mean liver iron concentration and BCS of the supplemented group at the start of the study was 258.6 ± 9.9 mg/kg of DM and 3.2 ± 0.16, respectively, for the treatment group and 278.1 ± 16.0 mg/kg of DM and 3.1 ± 0.26, respectively, for the control group. Both groups were fed identical diets formulated to meet the dietary requirements for net energy for lactation, protein, and minerals according to Dutch standards (CVB, 2005). Before calving, heifers were offered grass silage for ad libitum intake, 1.7 kg of DM corn silage, and 100 g of a mineral mixture for dry cows containing no added vitamin E or Se. After calving, heifers received a diet containing grass silage for ad libitum intake, 2.6 kg of DM corn silage, and concentrates. The grass silage fed prepartum had an OM digestibility of 70.4%, and contained 217 μg of Se and 8.5 IU of vitamin E per kg of DM, whereas the grass silage fed postpartum had an OM digestibility of 74.4%, and contained 39 μg of Se and 20.9 IU of vitamin E per kg of DM. The corn silage fed throughout the entire experiment had an OM digestibility of 74.7% and contained 32 μg of Se per kg of DM. To estimate the total vitamin E intake, we assumed a concentra-
Measurements and Sampling

Animal Experiments of Utrecht University. The experiment finished at 8 wk postpartum and was approved by The Ethical Committee for animal experiments. The control group received the corn-based carrier without carrier) to the vitamin E-supplemented group. The IU vitamin E (as α-tocopherol acetate in a corn-based carrier) to the vitamin E-supplemented group. The control group received the corn-based carrier without vitamin E. The experiment finished at 8 wk postpartum and was approved by The Ethical Committee for Animal Experiments of Utrecht University.

**Measurements and Sampling**

Individual feed intake was measured daily from 3 wk before calving until the end of the experiment. Throughout the experiment, BCS was scored weekly by the same trained observer using the system developed by Ferguson et al. (1994), wherein the scorer is directed to view certain anatomical sites of the pelvic and loin area. It uses a scale from 1 (emaciated) to 5 (fat) in 0.25-point increments. Heifers were milked twice daily and milk yield was recorded at each milking. A peroxidation marker, d-ROM, and a set of antioxidants were measured. The antioxidants were SOD and glutathione peroxidase (GSH-Px) activity in red blood cells (RBC), reduced glutathione (GSH) and oxidized glutathione (GSSG) concentration in RBC, vitamin E, and protein thiol groups (pSH). All variables were measured in blood, and specific oxidants and antioxidants were also measured in liver tissue, milk, and ovarian follicular fluid. Data on MDA in blood, liver, and milk have been presented separately (Bouwstra et al., 2005). On the day of calving, all heifers received 2 kg of concentrates, which was gradually increased so that the allocated amount of 7 kg/d was achieved by 18 d postpartum. Concentrates were fed as pellets (4.5% crude fat, 9.6% starch, and 300 μg of Se on a DM basis) and included a mineral mixture without added vitamin E. Corn silage and concentrates were offered in equal portions twice daily. The grass silage was available during the remainder of the day and was offered twice daily after feeding corn silage. Fresh water was available at all times. Heifers were placed in the experimental barn and the prepartum diet was fed 1 wk before the experiment started to adapt to the housing and feeding conditions of the experiment. The experimental treatment started at 8 wk before the expected calving date with daily oral supplementation of 3,000 IU vitamin E (as α-tocopherol acetate in a corn-based carrier) to the vitamin E-supplemented group. The control group received the corn-based carrier without vitamin E. The experiment finished at 8 wk postpartum and was approved by The Ethical Committee for Animal Experiments of Utrecht University.

**Liver Tissue.** In wk −9, liver biopsies were taken for determination of iron concentration. These samples were immediately transported to the laboratory for analysis. Additional liver biopsies were taken after blood sampling in wk −5, −1, 2, and 8 postpartum. Before sampling, the biopsy area was shaved, disinfected, and anesthetized with 7 mL of lidocaine with epinephrine (Alfacaine 2%, Alfasan, Woerden, the Netherlands). A stab incision was made in the right 11th intercostal space at the level of the greater trochanter. Between 2 sampling sessions, the place of sampling was varied to avoid repeated sampling of the same liver site. After sampling, the biopsy was macroscopically checked for representative liver tissue. Approximately 400 mg (wet weight) of liver tissue was collected with a 17G × 200 mm biopsy needle. The sampling method did not cause noticeable discomfort for the cows involved, although a slight pain reaction could be noticed only when the biopsy needle passed through the peritoneum.

**Milk.** Milk samples were collected from every milking during the first wk (d 0 to 8) and on d 14 during the morning milking for determination of vitamin E, MDA, fat, protein content, and SCC. Samples were stored at −80°C before analysis.

**Ovarian Follicular Fluid.** A synchronization protocol (Crestar+ method, Intervet, Boxmeer, the Netherlands) was used to synchronize estrous cycles and ovarian structures for the subsequent harvest of ovarian follicular fluid. At 5 to 6 wk postpartum, heifers received a Crestar ear implant containing 3 mg of norgestomet and an intramuscular prostaglandin injection. After 8 d, another prostaglandin injection was administered, and 2 d later the ear implant was removed. Follicular growth was monitored by measuring follicular size using daily ultrasound observations (type SSD-210 DX, Linear Array 7.4 MHz, Aloka, Tokyo, Japan). Two days after removal of the ear implant, follicles measuring >15 mm in diameter were regarded as preovulatory (Lucy et al., 1992) and were punctured transvaginally via ultrasound guidance. Epidural anesthesia with 2 mL of lidocaine with epinephrine (Alfacaine 2%, Alfasan) was provided before collection. Only ovarian follicular fluid samples without any visible blood contamination were stored at −80°C until analysis for concentrations of vitamin E, MDA, and NEFA and activity of GSH-Px.

Blood. Blood samples were taken from the jugular vein in 7-d intervals at 0800 h except in wk −2, −1, and 0, when samples were taken twice weekly. Blood was collected in an evacuated tube containing lithium heparin as an anticoagulant (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) for the collection of RBC. This sample was centrifuged (10 min at 2,000 × g and 4°C) and washed 3 times in PBS. Harvested RBC were stored at −80°C for analyses of SOD and GSH-Px activity and GSH and GSSG concentrations. Serum separator tubes (Vacutainer, Becton Dickinson) without anticoagulant were used to obtain blood serum. After collection, they were centrifuged for 15 min at 3,500 × g at 4°C, and serum was stored at −80°C for analyses of vitamin E, cholesterol, d-ROM, pSH, and NEFA.
**Laboratory Analyses**

For determination of liver iron concentration, fresh liver samples (200–250 mg) were homogenized and destroyed in an HNO₃ solution. Iron concentration was determined with an inductively coupled plasma atomic emission spectroscopy (ICP-AES) technique (NEN-EN 15510; NEN, 2007). All other liver samples (~150–200 mg) were frozen and stored in liquid nitrogen, immediately after collection. Once all the samples of the experiment were taken, the liver samples were thawed, homogenized in PBS (5:1), centrifuged at 15,800 × g at 4°C, and refrozen at −80°C until analysis of vitamin E, MDA, GSH-Px, triacylglycerol (TAG), and total protein. Concentrations were expressed per gram of protein to correct for differences in sample weight and dissolution.

Analyses of cholesterol, TAG, hemoglobin, and total protein were performed using a clinical auto analyzer (Hitachi 912, Roche Diagnostics, Almure, the Netherlands) and commercial kits (Roche Diagnostics). Activity of GSH-Px was measured on the same auto analyzer using a Ransel kit (Randox, Antrim, UK). Total GSH and GSSG were measured after deproteinization by using glutathione reductase and 5,5-dithiobis-2-nitrobenzoyl acid (DTNB). Activity of GSSG was determined after derivatization of GSH by 2-vinylpyridine on an autoanalyser (Griffith, 1980). Glutathione and the ratio GSH:GSSG were calculated from these values. The determination of pSH groups was conducted using DTNB, which reacts specifically with thiol groups to give a highly colored yellow anion that has an absorbance peak at 405 nm (Hu, 1994). Malondialdehyde in ovarian follicular fluid was measured with an isocratic Varian HPLC from Chromosystems (Munich, Germany). A 10-cm C18 cartridge (Varian) was used with a flow rate of 1.0 mL/min at ambient temperature (25°C). Fluorescence detection occurred with excitation at 515 nm and emission at 553 nm. Vitamin E (α-tocopherol) was measured by HPLC with both UV and fluorescence detection using a kit from Chromosystems. Determinable reactive oxygen metabolites were determined spectrophotometrically with the chromogenic substrate N,N-dimethyl-p-phenylenediamine.

Concentrations of fat and protein in milk samples were determined with mid infrared spectrometry (MilkoScan 4000 or MilkoScan FT6000, Foss, Hillerød, Denmark) according to standard method 141C (IDF, 2000).

Selenium concentration in forages was determined with inductively coupled plasma–mass spectroscopy (ICP-MS) technique (ISO 17294-2; ISO, 2003) after microwave digestion with nitric acid. The α-tocopherol concentration in the 2 grass silages was measured in one representative sample of each silage stack according to the method approved by Analytical Methods Committee (1991). Briefly, the sample was hydrolyzed with an ethanolic potassium hydroxide solution and α-tocopherol was extracted into light petroleum. After evaporation of the petroleum, the residue was dissolved in hexane. The α-tocopherol content of the sample extract was determined using HPLC.

**Statistical Analysis**

Effects of vitamin E supplementation on blood, liver, and milk variables, and BCS were analyzed by repeated-measures ANOVA (PROC MIXED of SAS, version 9.1; Littell et al., 2006). Heifer was included as the repeated subject. Treatment (supplemented vs. control), week (−8 to 8 postpartum), and their interaction were included as fixed effects in the model. For blood variables, linear and quadratic effects were compared (from 8 wk prepartum until 8 wk postpartum). If quadratic effects were significant, interaction terms (time × time and time × time × treatment) were included. If not significant, they were omitted from the model. In addition, baseline values of the dependent variables were used to covariately adjust the data. The baseline value was defined as the value of the dependent variable at 8 wk (for blood variables and BCS) or 5 wk (for liver variables) before calving. The baseline value for serum NEFA concentration was set at 2 wk prepartum. Milk production data (only measured at 1, 2, and 8 wk postpartum) were not corrected for a baseline value. Based on a comparison of covariance structures (unstructured, compound symmetry, autoregressive order 1, Huynh-Feldt, and Toepplitz) and best-fit statistics as evaluated by Akaike’s information criterion and Schwarz’s Bayesian criterion, autoregressive order 1 was selected as the covariance structure for submatrices for each variable. If main effects were detected, a post-hoc Student’s t-test was used to localize effects in time series.

Variables in ovarian follicular fluid (i.e., only at 8 wk postpartum), which were not repeatedly measured, were analyzed by PROC GLM (version 9.1, SAS Institute) including only treatment (supplemented vs. control) as a fixed effect.

If treatment effects were not detected, BCS at 4 wk before calving (i.e., when the periparturient metabolic changes have not started yet; Mulligan and Dohert, 2008) and changes in BCS with time were related to changes in blood variables from 4 wk before until 8 wk after calving. At 4 wk before calving, 7 heifers had a BCS <3.0 and 9 had a BCS ≥3.0. First, the initial BCS (at 4 wk prepartum) and slope for BCS with time were assessed for all heifers individually, and the slope for blood variables with time was calculated for each individual heifer. Second, a linear regression analysis
was performed to determine a relationship between the initial value and slope of BCS and change in blood variables. A similar analysis was done for the increase in milk yield after calving, because both BCS and milk production were hypothesized to relate to the metabolic rate and could therefore affect oxidative stress in periparturient heifers (Bernabucci et al., 2005; Löhrke et al., 2005; O’Boyle et al., 2006).

Correlations between variables were calculated as partial correlation coefficients corrected for treatment and time effect, except for ovarian follicular fluid, which was adjusted for treatment only. Partial correlation coefficients of MDA in blood and liver with other parameters are reported in this paper, because they were not reported in a separate paper (Bouwstra et al., 2005; O’Boyle et al., 2006).

All data were evaluated using SAS software (version 9.1; SAS Institute Inc., Cary, NC). Effects were considered significant at \( P < 0.05 \) and a tendency was detected at \( P < 0.10 \). Data are presented as least squares means ± SEM.

RESULTS

Heifer Health and Milk Production

Two heifers, one in the supplemented group and one in the control group, were removed from the experiment because of a displaced abomasum. Other health problems were not observed. Therefore, 16 animals completed the study, and all blood, liver, and milk samples were successfully collected.

Daily milk yield increased during the experiment to a peak yield of 25.9 ± 0.96 kg/d in wk 8 and was not different between the groups at any point in time. Milk fat (6.15 ± 0.22%) and protein (4.06 ± 0.07%) content did not differ between groups. The milk yield at 1 wk after calving was negatively associated with GSSG (\( P = 0.041 \)) in RBC. Daily milk yield at 1 wk after calving and the increase in milk yield were negatively associated with GSH-Px activity in liver (\( P = 0.026 \)). The milk yield at 1 wk after calving and the subsequent increase in milk yield from wk 1 to 8 was not correlated with any other of the components in blood, liver, or ovarian follicular fluid (results not shown).

Selenium and Vitamin E Intake

For both treatment groups, the estimated Se intake was 1.1 mg of Se/d during the last 4 wk prepartum. During lactation, Se intake was initially 0.92 mg/d and gradually increased to 2.4 mg/d when maximum concentrate intake was achieved. The estimated vitamin E intake of the control group was 105.1 IU/d during the last 4 wk prepartum and 459 IU/d during the 8 wk postpartum.

Blood Variables

Responses of blood vitamin E, cholesterol, NEFA, oxidants, and antioxidants to vitamin E supplementation are presented in Table 1. Supplementation increased (\( P < 0.05 \)) vitamin E, the vitamin E to cholesterol ratio, NEFA, and pSH, but did not affect other variables in blood.

Serum NEFA concentration was higher (\( P = 0.016 \)) for the supplemented heifers and increased (\( P < 0.001 \)) with time. Compared with the baseline value, serum NEFA concentration increased in the week of calving for both treatment groups (\( P < 0.01 \)). After calving, serum NEFA concentration was higher than the baseline value in wk 1 for control heifers (\( P = 0.021 \)) and in wk 2 for supplemented heifers (\( P = 0.009 \)). There was a tendency (\( P = 0.070 \)) for d-ROM to be higher in the supplemented heifers. In the supplemented heifers, d-ROM values increased significantly 2 wk postpartum, compared with the baseline value (\( P = 0.0259 \)).

A time effect was observed for all variables except for GSSG and SOD (Table 1).

Serum MDA did not correlate with serum d-ROM (\( r = −0.04, \ P = 0.557 \)) or with any of the antioxidants measured in serum. Serum d-ROM was positively correlated with GSH-Px (\( r = 0.39, \ P = 0.001 \)), but not with SOD (\( r = 0.11, \ P = 0.343 \)). Serum d-ROM also correlated with serum GSSG (\( r = 0.42, \ P < 0.001 \)), with GSH;GSSG (\( r = 0.30, \ P = 0.008 \)), and with pSH (\( r = −0.363, \ P = 0.001 \)). Serum d-ROM was positively correlated with liver protein-adjusted TAG (\( r = 0.52, \ P = 0.002 \)), but not with serum NEFA.

Liver Variables

Supplemented heifers had higher liver protein-adjusted TAG concentrations (\( P = 0.040 \)) (see Table 2) than did control heifers. For both groups, the TAG concentration was highest at 2 wk after calving and decreased afterward, but it remained higher than the level before calving. Liver TAG concentration was correlated with serum NEFA concentration (\( r = 0.66, \ P < 0.001 \)), but not with liver protein-adjusted MDA. Activities of GSH-Px and SOD in liver did not differ between the supplemented and control groups. In both the supplemented and control groups, activity of GSH-Px was significantly lower 2 wk postpartum compared with the baseline value at 8 wk prepartum. The activity of SOD was significantly lower 2 wk postpartum in the supplemented group. Liver vitamin E concentration was positively correlated to liver GSH-Px activity (\( r = 0.58, \)}
but not with liver SOD. Serum d-ROM was negatively correlated with liver vitamin E \((r = -0.45, P = 0.009)\) and positively with protein-adjusted liver TAG \((r = 0.52, P = 0.002)\).

### Ovarian Follicle Variables

At the end of the synchronization period, 9 heifers showed a large follicle with a diameter >15 mm, which was considered a preovulatory sized follicle and could be sampled. Supplemented heifers were, on average, 52.6 ± 0.9 d and control heifers 53.5 ± 1.4 d in lactation when ovarian follicular fluid was collected. Vitamin E concentration was higher \((P = 0.008)\) in ovarian follicular fluid derived from supplemented heifers compared with control heifers (Table 3). The level of vitamin E in ovarian follicular fluid was 2 to 3 times lower than that in blood. Activity of GSH-Px in ovarian follicular fluid tended to be lower \((P = 0.087)\) in the supplemented group. Other oxidative markers in the ovarian follicular fluid did not differ between the 2 groups. Neither milk yield at 1 wk after calving nor the increase in milk yield from calving until 2 wk after calving was associated with oxidative markers in ovarian follicular fluid.

### BCS

Body condition score decreased with time \((P < 0.001)\) but this decrease did not differ \((P = 0.877)\) between treatment groups. For all individual heifers except one, BCS decreased \((P < 0.001)\) from wk 4 prepartum until wk 8 postpartum, with an average for 15 heifers of −0.08 ± 0.01 units/wk. One heifer, which had a very low BCS (1.75) at the start of the experiment, did not show a further decrease in BCS during the study. Body condition score loss from 4 wk before calving to 8 wk after calving was greater \((P < 0.001)\) for the high-BCS \((\geq 3)\) heifers \((-0.12 \pm 0.01\) unit/wk) than for the low-BCS \((<3)\) heifers \((-0.05 \pm 0.01\) unit/wk). For all oxidative stress markers, effects of vitamin E supplementation were not affected by initial BCS. The BCS at 4 wk before calving and the BCS loss during the following 12 wk were related to changes in blood variables (Table 4). A higher BCS and more BCS loss were associated with a tendency for a higher d-ROM \((P = 0.096\) and 0.059, respectively). The interaction between BCS and BCS loss on d-ROM was significant \((P = 0.036)\). The serum MDA concentration and the activity of GSH-Px and SOD in RBC were not affected by initial BCS or BCS loss. Change of BCS and the interaction between BCS and BCS change were significantly correlated with GSH, GSSG, and their ratio in the RBC. A higher BCS at 4 wk before calving was associated with a higher ratio of GSH to GSSG \((P = 0.047)\). The concentration

### Table 1. Effect of vitamin E supplementation (+Vit E) on vitamin E, cholesterol, NEFA, and antioxidants in blood of periparturient heifers from 8 wk prepartum to 8 wk postpartum

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment (Trt)</th>
<th>Parameter estimates (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E, (\mu M)</td>
<td>+Vit E</td>
<td>6.11 (0.67)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>11.20 (0.67)</td>
</tr>
<tr>
<td>NEFA, (\mu M)</td>
<td>+Vit E</td>
<td>0.66 (0.07)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.90 (0.07)</td>
</tr>
<tr>
<td>Plasma protein thiols, (\mu M)</td>
<td>+Vit E</td>
<td>281 (7)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>305 (7)</td>
</tr>
<tr>
<td>Cholesterol, (\mu M)</td>
<td>+Vit E</td>
<td>2.10 (0.11)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2.10 (0.11)</td>
</tr>
<tr>
<td>Reactive oxygen metabolites, (U/mL)</td>
<td>+Vit E</td>
<td>84.4 (3.9)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>84.4 (3.9)</td>
</tr>
<tr>
<td>Glutathione peroxidase, (U/mmol Hb)</td>
<td>+Vit E</td>
<td>2.627 (7.19)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1.127 (7.19)</td>
</tr>
<tr>
<td>Superoxide dismutase, (U/mmol Hb)</td>
<td>+Vit E</td>
<td>0.07 (0.02)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.07 (0.02)</td>
</tr>
<tr>
<td>Glutathione (GSH), (\mu mol/mmol Hb)</td>
<td>+Vit E</td>
<td>0.08 (0.03)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.08 (0.03)</td>
</tr>
<tr>
<td>Glutathione disulfide (GSSG), (\mu mol/mmol Hb)</td>
<td>+Vit E</td>
<td>0.08 (0.03)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.08 (0.03)</td>
</tr>
<tr>
<td>Vitamin E: cholesterol, (\mu mol/mmol)</td>
<td>+Vit E</td>
<td>4.17 (0.23)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>4.17 (0.23)</td>
</tr>
<tr>
<td>GSH:GSSG, (mol/mol)</td>
<td>+Vit E</td>
<td>−0.05 (0.02)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>−0.05 (0.02)</td>
</tr>
</tbody>
</table>

Parameter estimates \(2\) for the regression model \(Y = A + B \cdot \text{time} + C \cdot \text{time} \cdot \text{time}\). If treatment or (quadratic) time effects were absent, those variables were omitted from the model \((n = 8\) if treatment effect was significant; \(n = 4\) if treatment effect was absent).

1 Activity of glutathione peroxidase, superoxide dismutase, and the concentrations of GSH, GSSG, and their ratio were measured in RBC.

2 Parameter estimates (SEM) for the regression model \(Y = A + B \cdot \text{time} + C \cdot \text{time} \cdot \text{time}\). If treatment or (quadratic) time effects were absent, those variables were omitted from the model \((n = 8\) if treatment effect was significant; \(n = 4\) if treatment effect was absent).
of GSSG and the GSH to GSSG ratio did not change in time for low- or high-BCS heifers. Blood pSH was not affected by BCS at 4 wk before calving nor by the change in BCS. The BCS at 4 wk before calving and BCS change were not related to liver TAG or NEFA concentrations.

DISCUSSION

This experiment studied the effects of a dietary supplementation of 3,000 IU of vitamin E per d from 8 wk prepartum until 8 wk postpartum on markers of oxidative damage and several antioxidants in dairy heifers. The oxidative status was assessed from multiple sampling sites: blood, liver, and ovarian follicle fluid. In addition, the prepartum BCS (i.e., 4 wk before calving) and the change in BCS until wk 8 postpartum were associated with oxidative stress markers.

Effects of Vitamin E Supplementation

In contrast to our hypothesis, daily supplementation with 3,000 IU of vitamin E did not reduce d-ROM in serum and MDA in ovarian follicular fluid. This does not correspond with the reduction of MDA in blood at 2 wk after calving and in liver at 2 and 8 wk after calving upon vitamin E supplementation (Bouwstra et al., 2008). Malondialdehyde is derived mainly from fatty acids with 2 or more double bounds (Södergren, 2000), whereas d-ROM is a marker for hydroperoxides, which are breakdown products of lipids as well as of other organic substrates (Yamanaka et al., 2006). This may indicate that vitamin E supplementation inhibited peroxidation of fatty acids with 2 or more double bonds in the liver, but did not affect peroxidation of more-saturated fatty acids and other organic compounds in the liver or other organs of periparturient heifers under the given nutritional circumstances. Indeed, it has been shown that specific tissues may differ in oxidative status (Jenkinson et al., 1999).

At a Se intake below actual NRC requirements, vitamin E supplementation, and not Se supplementation, reduced the concentration of TBARS in RBC (Brzezinska-Slebodzinska et al., 1994). Effects of supplementation of vitamin E on variables related to oxidative stress depend on the quantity of vitamin E that is supplemented (Weiss et al., 1997) and on the baseline vitamin E status (LeBlanc et al., 2002; Persson Waller et al., 2007). In the current study, vitamin E intake was 105.1 IU/d prepartum and 459 IU/d postpartum,

| Table 2. Effect of vitamin E supplementation (+Vit E) on vitamin E, cholesterol, triacylglycerides (TAG), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) in liver of periparturient heifers from 5 wk prepartum to 8 wk postpartum. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Treatment (Trt)** | **Control** | **+Vit E** | **P-value** |
| **Variable** | **Trt** | **Time** | **Trt × Time** |
| Vitamin E, μM | 0.057 ± 0.003 | 0.078 ± 0.003 | 0.002 <0.001 0.342 |
| Cholesterol, mM | 0.019 ± 0.001 | 0.017 ± 0.001 | 0.342 0.072 0.564 |
| TAG, mM | 0.098 ± 0.008 | 0.125 ± 0.008 | 0.040 <0.001 0.014 |
| GSH-Px, U/mg of protein | 94.8 ± 4.0 | 91.8 ± 4.0 | 0.620 <0.001 0.911 |
| SOD, U/mg of protein | 22.56 ± 0.76 | 21.20 ± 0.76 | 0.224 <0.001 0.631 |

1Values are least squares means ± SEM (n = 8 for each group).

| Table 3. Effect of vitamin E supplementation (+Vit E) on follicle size, and concentration or activity of vitamin E, cholesterol, NEFA, malondialdehyde (MDA), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), triacylglycerides (TAG), and total protein in follicular fluid of heifers 6 to 8 wk postpartum. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Treatment** | **Control** | **+Vit E** | **P-value** |
| **Variable** | **Follicle size, mm** | **Vitamin E, μM** | **Cholesterol, mM** | **NEFA, mmol/L** | **TAG, mmol/L** | **Total protein, g/L** | **MDA, μg/L** | **GSH-Px, U/mg of protein** | **SOD, U/mg of protein** |
| Follicle size, mm | 18.3 ± 1.5 | 18.0 ± 2.5 | 0.868 |
| Vitamin E, μM | 6.98 ± 0.77 | 10.80 ± 0.69 | 0.008 |
| Cholesterol, mM | 1.86 ± 0.15 | 1.77 ± 0.15 | 0.690 |
| NEFA, mmol/L | 0.77 ± 0.11 | 0.74 ± 0.12 | 0.865 |
| TAG, mmol/L | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.648 |
| Total protein, g/L | 66.7 ± 2.1 | 62.1 ± 2.1 | 0.165 |
| MDA, μg/L | 12.55 ± 2.06 | 13.85 ± 1.84 | 0.654 |
| GSH-Px, U/mg of protein | 57.8 ± 9.4 | 39.4 ± 8.4 | 0.187 |
| SOD, U/mg of protein | 0.27 ± 0.02 | 0.21 ± 0.02 | 0.087 |

1Values are least squares means ± SEM (n = 4 for the control group; n = 5 for the supplemented group).
which is below the NRC (2001) requirements. Indeed, the low vitamin E status in the heifers was confirmed by a mean serum α-tocopherol concentration of 1.69 μg/mL of serum in the 4 wk before parturition, whereas serum plasma α-tocopherol at parturition should be at least 2.0 μg/mL of serum (Herdt and Stowe, 1991).

Selenium intake met the Dutch requirement for Se during the prepartum period and was above the requirement in the postpartum period (CVB, 2005). Selenium status can be monitored by measuring the GSH-Px activity in RBC (Knowles et al. 1999). The lowest GSH-Px activity (2,554 ± 260.3 U/mmol of Hb) was found at 8 wk after calving, which is above the threshold value of 1,930 U/mmol of Hb used in the Netherlands but lower than the threshold of 4,025 U/mmol of Hb suggested by Guyot et al. (2009). This suggests that Se status meets Dutch standards but could still be marginal.

Vitamin E supplementation did not affect SOD and GSH-Px activity in RBC in the current study. In general, effects of vitamin E supplementation on the oxidant/antioxidant system in dairy cows are inconclusive. Brzezinska-Slebodzinska et al. (1994) found that daily supplementation with 1,000 IU of vitamin E increased GSH and tended to increase GSH-Px activity. In that experiment, Se intake was also below actual NRC (2001) requirements. However, Bourne et al. (2007) did not find effects of parenteral or oral vitamin E supplementation on GSH-Px activity in RBC in dry and lactating cows. A recent field study, where a mineral mixture including trace minerals plus 10,000 IU of vitamin E/d was supplemented to dry cows, also did not show an effect on SOD and GSH-Px activity in the liver in combination with 2 fat sources (lard or fish oil) and various levels of iron intake in rats (Ibrahim et al., 1997).

Vitamin E–supplemented heifers had higher serum NEFA and higher protein-adjusted liver TAG concentrations than control heifers. This suggests more fat mobilization in the supplemented heifers, but there were no differences in milk fat content and BCS loss between treatment groups. Serum NEFA and liver TAG concentrations correspond with those commonly found in dairy cows (Van de Top et al., 1996; Vandehaar et al., 1999) indicating that excessive fat mobilization or hepatic steatosis did not occur. Causes for the treatment effect on serum NEFA and liver TAG therefore remain unclear.

Table 4. Association of the BCS at 4 wk prepartum until 8 wk postpartum on changes in cholesterol, NEFA, oxidants, and antioxidants in the blood of periparturient heifers1,2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Intercept</th>
<th>Slope</th>
<th>P-value</th>
<th>Intercept</th>
<th>Slope</th>
<th>Intercept × slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol, mmol/L</td>
<td>0.21 ± 0.09</td>
<td>−1.30 ± 2.19</td>
<td>0.037</td>
<td>0.564</td>
<td>0.232</td>
<td></td>
</tr>
<tr>
<td>GSH-Px, U/mmol of Hb</td>
<td>104 ± 88</td>
<td>1,216 ± 2,178</td>
<td>0.258</td>
<td>0.587</td>
<td>0.609</td>
<td></td>
</tr>
<tr>
<td>SOD, U/mmol Hb</td>
<td>245 ± 947</td>
<td>3,571 ± 23,473</td>
<td>0.800</td>
<td>0.882</td>
<td>0.886</td>
<td></td>
</tr>
<tr>
<td>Glutathione (GSH), μmol/mmol of Hb</td>
<td>3.11 ± 1.59</td>
<td>−110 ± 39</td>
<td>0.074</td>
<td>0.017</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>Glutathione disulfide (GSSG), mol/mol of Hb</td>
<td>−0.65 ± 0.61</td>
<td>49.7 ± 15.1</td>
<td>0.310</td>
<td>0.006</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>Reactive oxygen metabolites, U/mL</td>
<td>2.63 ± 1.45</td>
<td>−75.4 ± 36.0</td>
<td>0.099</td>
<td>0.059</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td>Malondialdehyde, μg/L</td>
<td>0.42 ± 0.25</td>
<td>−9.40 ± 6.22</td>
<td>0.119</td>
<td>0.157</td>
<td>0.163</td>
<td></td>
</tr>
<tr>
<td>Protein thiols, μmol/L</td>
<td>6.31 ± 3.40</td>
<td>−79.0 ± 84.3</td>
<td>0.088</td>
<td>0.368</td>
<td>0.253</td>
<td></td>
</tr>
<tr>
<td>NEFA, mmol/L</td>
<td>0.11 ± 0.06</td>
<td>−1.53 ± 1.51</td>
<td>0.105</td>
<td>0.350</td>
<td>0.195</td>
<td></td>
</tr>
<tr>
<td>GSH:GSSG, μmol/μmol</td>
<td>0.0001 ± 0.0001</td>
<td>0.001 ± 0.001</td>
<td>0.047</td>
<td>0.006</td>
<td>0.007</td>
<td></td>
</tr>
</tbody>
</table>

1Values are least squares means (±SE).
2Activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) and the concentrations of glutathione, glutathione disulfide, and their ratio are measured in RBC.

To the authors’ knowledge, this is the first study reporting oxidant and antioxidant concentrations in bovine ovarian follicular fluid. The level of vitamin E in ovarian follicular fluid was 2 to 3 times lower than in blood. Vitamin E supplementation increased vitamin E concentration in the follicles by 55% and numerically, but not significantly (only 9 heifers were used for sampling follicular fluid), reduced MDA concentrations in the follicles by 32%.

**Time Effects**

The higher serum d-ROM concentration 2 wk after calving observed in the supplemented heifers indicates oxidative damage after calving. However, this finding contradicts that of Bernabucci et al. (2005), who observed a dramatic decrease in d-ROM concentration around calving.

Activities of SOD and GSH-Px in RBC and in liver were not increased around calving, although several
studies have reported a higher SOD and GSH-Px activity in RBC during the periparturient period in dairy cows (Bernabucci et al., 2005; Stefanon et al., 2005; Colitti and Stefanon, 2006; Gaál et al., 2006). Increased activity of SOD and GSH-Px is considered indicative for oxidative stress. Hence, this study does not indicate increased oxidative stress around calving in heifers.

Glutathione in RBC was lower around calving for both treatment groups. This decrease might be caused by increased use or reduced synthesis of GSH or a combination of both. However, GSH:GSSG did not change significantly during the experiment. A decrease in GSH:GSSG reflects the rate of removal of reactive molecules such as hydrogen peroxide (Skalicky et al., 2008). Like GSH, pSH contains thiol groups. The pSH concentration remained constant throughout the experimental period, which is in contrast to the study of Bernabucci et al. (2005), which found an increase in pSH before calving followed by a decrease between 5 and 10 d after calving.

The antioxidants (activity of SOD and GSH-Px and pSH concentration) measured in this study and GSH:GSSG remained virtually constant around calving, which suggests that these heifers did not undergo oxidative stress in the periparturient period. Moreover, MDA in ovarian follicular fluid did not differ between treatment groups. Only the concentrations of d-ROM showed temporary increases in serum and indicated that some oxidative stress related to the metabolic pathways associated with this marker may occur. Löhrke et al. (2005) reported a positive and almost linear relationship between milk production and the concentration of serum lipid hydroperoxides in LDL. Therefore, the lower than expected milk production may have concomitantly resulted in an increased release of ROS such as the superoxide anion. In the current study in heifers, milk yield may have been too low to find a relationship between milk production and oxidative stress. The relatively low intake of the grass silage before calving and during lactation may explain the low milk production.

**Association of BCS and Oxidative Damage**

**Initial BCS.** In contrast to our hypothesis, there was no interaction between initial BCS and the effect of vitamin E supplementation on oxidative stress markers. Moreover, differences in BCS loss were not affected by vitamin E supplementation. Therefore, we have associated BCS and BCS loss with changes in oxidative status. Only serum d-ROM tended to be higher for heifers with a higher BCS, which was not found for serum MDA. More lipid peroxidation was expected at a higher BCS, because more visceral fat is associated with more oxidative damage in humans (Palmieri et al., 2006; Skalicky et al., 2008) and in fatter cows (Bernabucci et al., 2005). Moreover, O’Boyle et al. (2006) concluded that fatter cows had more oxidative stress and this conclusion was based on a lower blood GSH:GSSG. A decrease in GSH:GSSG is one of the markers of oxidative processes. However, the heifers in this study with a higher BCS had a slightly higher GSH:GSSG, reflecting a slight decrease in this oxidative process. Hence, these results do not suggest that a higher BCS is associated with more oxidative stress.

**BCS Loss.** A higher BCS at calving is associated with a higher BCS loss after calving (Holtner, 1990); thus, effects of initial BCS and BCS loss are interdependent. Loss of BCS in the last month of gestation was also reported by Bernabucci et al. (2005). In the current study, heifers that lost more body condition had the level of peroxidation of lipids and other organic substrates in the liver.

**Milk Production**

Vitamin E supplementation did not affect milk yield or milk composition, which corresponds with findings from other studies (Weiss et al., 1990; Baldi et al., 2000; Politís et al., 2004) in which no effects of vitamin E supplementation on milk yield and milk composition were found. In addition, milk production did not correlate with any of the oxidants or antioxidants. Other studies have shown a relationship between daily milk yield and lipid hydroperoxides in low-density lipoprotein (Löhrke et al. 2005) and only SOD activity in plasma (Wullepit et al., 2009). A higher milk yield was expected to result in an increased metabolic rate, which may concomitantly result in an increased release of ROS such as the superoxide anion. In the current study in heifers, milk yield may have been too low to find a relationship between milk production and oxidative stress. The relatively low intake of the grass silage before calving and during lactation may explain the low milk production.

**Correlations**

In contrast to serum MDA, serum d-ROM was significantly correlated with some antioxidants. Plasma d-ROM correlated positively with the activity of GSH-Px and with GSH:GSSG. This corresponds with the study of Gabai et al. (2004) in dairy heifers in which GSH-Px was also positively related to peroxidation products, as measured by TBARS instead of d-ROM. Plasma d-ROM was negatively correlated with plasma pSH in the current study, which corresponds with a negative correlation between TBARS and SH in plasma of dairy cows (Tanaka et al., 2007; Bouwstra et al., 2009). Protein-adjusted liver TAG was positively correlated with serum d-ROM, but this correlation was strongly influenced by 3 leverage points. Therefore, serum d-ROM would not be an accurate marker to estimate the level of peroxidation of lipids and other organic substrates in the liver.
higher concentrations of reduced GSH, lower GSSG, and a higher GSH:GSSG in blood. They also tended to have higher serum d-ROM. This trend is opposite to the higher GSH:GSSH in blood as discussed before. These findings do not confirm that BCS loss, as such, induces more oxidative damage.

CONCLUSIONS

Despite the low vitamin E status and marginal Se status of these heifers, vitamin E supplementation did not reduce d-ROM concentration in blood or MDA concentration in ovarian follicular fluid. The measured antioxidants did not change significantly in serum and liver during the experimental period and do not suggest oxidative processes in the periparturient period of these heifers. This might be caused in part by low milk yield. Milk yield was not associated with peroxidative damage or with most antioxidants. Cows with a BCS of ≥3 are hardly prone to more peroxidative damage.

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