The effect of a negative energy balance status on β -carotene availability in serum and follicular fluid of nonlactating dairy cows

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ABSTRACT

Maternal metabolic pressure due to a cow's negative energy balance (NEB) has a negative effect on oocyte quality as a result of increased oxidative stress. In this study, we hypothesized that a NEB status may negatively affect the availability of β -carotene (bC, an antioxidant) in the micro-environment of the oocyte or follicular fluid (FF) and that daily bC supplementation can increase bC availability. We aimed to (1) determine the effect of a nutritionally induced NEB on bC concentrations in serum and FF as well as on the presence of bC metabolites, oxidative stress levels, and follicular growth in a nonlactating dairy cow model, and (2) investigate how this effect could be altered by dietary bC supplementation. Six multiparous nonlactating Holstein Friesian cows were subjected to 4 consecutive dietary treatments, 28 d each: (1) $1.2 \times$ maintenance (M) or positive energy balance (PEB) without bC supplement (PEB-bC), (2) $1.2 \times$ M with daily supplement of 2,000 mg of bC comparable to the level of bC intake at grazing (PEB+bC), (3) $0.6 \times$ M with 2,000 mg of bC (NEB+bC), and (4) $0.6 \times$ M (NEB-bC). At the end of each treatment, estrous cycles were synchronized and blood and FF of the largest follicle were sampled and analyzed for bC, retinol, α -tocopherol, free fatty acids, estradiol, and progesterone. Serum cholesterol, triglycerides, urea, insulin growth factor 1, growth hormone, total antioxidant status (TAS), and red blood cell glutathione (GSH) concentrations were determined as well. All cows lost body weight during both energy restriction periods and showed increased serum free fatty acid concentrations, illustrating a NEB. A dietary induced NEB reduced FF bC, but not plasma bC or plasma and FF retinol concentrations. However, bC and retinol concentrations drastically increased in

both fluid compartments after bC supplementation. Follicular diameter was increased in supplemented PEB cows. Energy restriction reduced the TAS and red blood cell GSH, whereas daily bC supplementation could restore GSH concentrations, but not the TAS, to levels present in healthy PEB cows. In conclusion, daily bC supplementation can substantially improve bC and retinol availability in the oocyte's micro-environment, irrespective of the energy balance, which may affect follicular development and oocyte quality in the presence of maternal metabolic stress. This knowledge can be of importance to optimize nutritional strategies in the dairy industry to feed for optimal oocyte quality and fertility.

Key words: dairy cow, fertility, negative energy balance, β -carotene, follicular fluid

INTRODUCTION

The dairy industry has evolved drastically: due to decades of selection toward milk production, the modern high-yielding dairy cow produces up to 10,000 L of milk per lactation (CRV, 2012). This, together with a reduced appetite postpartum and thus reduction in DMI, contributes to the inability of a cow to compensate for the high loss of glucose through the udder. As a result, cows enter a period of negative energy balance (NEB) and increased metabolic stress that is associated with reduced fertility (Rukkwamsuk et al., 1999; Butler, 2003; Wathes et al., 2003; Drackley et al., 2005; Leroy et al., 2011). One important identified factor in the pathogenesis of subfertility during this period is the negative effect of metabolic stress due to the NEB (Bernabucci et al., 2005) on ovarian follicle and oocyte development (Beam and Butler, 1997; Kendrick et al., 1999; Dupont et al., 2014). During this NEB, upregulated lipolysis of fat stores results in increased serum free fatty acid (FFA) concentrations (Drackley et al., 2001). These FFA are reflected in the follicular fluid (FF; Leroy et al., 2004a), in which the oocyte matures, jeopardizing

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granulosa cell viability (Vanholder et al., 2005), oocyte developmental competence and subsequent embryo quality, and thus dairy cow fertility (Leroy et al., 2005; Van Hoeck et al., 2011, 2012). Gene expression analysis and functional assays of FFA-exposed oocytes, cumulus cells, and subsequent blastocysts pointed out the importance of pathways related to oxidative metabolism, redox status, and oxidative stress (OS; Van Hoeck et al., 2013).

The above described subfertility syndrome in modern dairying is a multifactorial problem in which not only genetics and management, but also nutrition, play a crucial role (Chagas et al., 2007). Substantial research has been performed on altering dairy cow nutrition to improve fertility (Santos et al., 2010; Leroy et al., 2014). In recent years, optimum vitamin nutrition in the dairy industry has gained more attention (for overview, see Calsamiglia and Rodríguez, 2012). Interest has been rising on dietary β -carotene (bC; provitamin A, precursor of retinol) because of its strong antioxidative properties and promising results on pregnancy rates in dairy cows following daily supplementation (Iwańska and Strusinska, 1997; Arechiga et al., 1998; Gossen and Hoedemaker, 2005; de Ondarza and Engstrom, 2009). Nowadays, dairy cows are more frequently housed under zero-grazing conditions in which cows are fed with conserved forages containing substantially lower bC levels than fresh grass (Chaveau-Duriot et al., 2005), emphasizing the rise of interest in the use of bC in the dairy industry. Considering the fact that OS is one of the main pathways by which elevated FFA affect bovine oocytes and embryos (Van Hoeck et al., 2013), bC might be a promising solution to ameliorate oocyte and embryo quality and thus dairy cow fertility. In contrast to the lack of in vitro research on bC and its effects on oocyte and embryo development, substantial in vivo research has been done investigating the effect of bC on dairy cow fertility. However, conflicting results about the actual effects of bC on dairy cow reproduction exist possibly due to (1) evaluation of reproductive performance by biased parameters as extensively reviewed by LeBlanc (2010); (2) the presence of bC in the control ration (Folman et al., 1983); (3) differences in the composition of the ration (Warner et al., 1970; Blomhoff et al., 1991; Yeum et al., 2000); (4) discrepancies in bC metabolism between different cow breeds (Bremel et al., 1982); and (5) differences in milk fat production and thus possible loss of fat-soluble bC through milk. Additionally, the most recent recommendations on bC supplementation in the dairy industry date from 2001 (NRC), and it is unlikely that these specific vitamin needs are still applicable to modern dairy cows (Barroeta et al., 2012). This stresses the need for more in-

depth information on bC supplementation and its effect on dairy cow reproduction.

Extensive research on the addition of antioxidants with the aim to optimize in vitro oocyte maturation and embryo culture media (reviewed in Premkumar and Agarwal, 2012) or to reduce reactive oxygen species in follicle cells, oocytes, and embryos shows beneficial effects (Pascoe et al., 1987; Olson and Seidel, 2000; Natarajan et al., 2010; Tao et al., 2010; Lin et al., 2013). It has been assumed that bC present in the FF can protect follicle cells and oocytes from reactive oxygen species (ROS)-mediated cytotoxicity (Ikeda et al., 2005). Moreover, in vitro research has shown that fatty acid-induced toxicity at the embryo level can be counteracted by the addition of antioxidants to the culture medium such as α -tocopherol (vitamin E; Rooke et al., 2012). Consequently, the antioxidant capacity of bC can possibly reduce the OS in the oocyte as a consequence of a NEB state. However, little is known about the availability of bC in the FF, the micro-environment of the maturing oocyte, and how oral supplementation can affect this. Furthermore, it can be hypothesized that the status of a NEB negatively affects serum and FF bC concentrations due to a higher systemic use (Sangsitavong et al., 2002). The NEB is primarily determined by a reduced DMI in the cow (Santos et al., 2010), rather than the increased energy loss through milk production. However, the significant rise in milk production can corroborate to the bC loss during NEB through milk. Consequently, a cow's milk production level and the concomitant metabolic stress status postpartum might complicate the understanding of how bC can reach the FF (Akar and Gazioglu, 2006; Calderón et al., 2007). A first important step that can contribute to clarify the effect of these confounders on the presence of bC in serum and FF is to unravel the underlying mechanisms in a fully controlled nonlactating dietary induced NEB cow model. As such we will investigate the effect of underfeeding and the subsequent NEB on the bC availability in blood and FF. Therefore, we aimed to (1) determine the effect of a nutritionally induced NEB on bC concentrations in serum and FF as well as on the presence of bC metabolites, OS levels, and follicular growth in a nonlactating dairy cow model, and (2) investigate how this effect could be altered by dietary bC supplementation.

MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich (Bornem, Belgium) unless specified otherwise and all compounds and reagents used for HPLC were of analytical grade. Results are shown as means $\pm$ SEM.

Table 1. Diet composition calculated for nonlactating Holstein Friesian cows with average BW of 600 kg

Item	PEB ¹ (1.2 × M)	NEB ¹ (0.6 × M)
Hay of average quality ² (kg of DM)	7.26	3.63
Chopped straw ³ (kg of DM)	1.90	0.95
Concentrate Bovimix 15% protein ⁴ (kg of DM)	1.55	0.77
Net energy (MJ/d)	±41.4	±20.7

¹PEB = positive energy balance; NEB = negative energy balance; M = maintenance.
²Hay contains 3,419 kJ of net energy per kg of DM.
³Straw contains 3,043 kJ of net energy per kg of DM.
⁴Concentrates contain 15% CP, 4% crude fat, 7.5% crude ash, and 14.5% crude cellulose on a DM basis and 6,964 kJ net energy per kg of DM (Quartes, Roeselaere, Belgium).

Animals and In Vivo Experimental Design

Six healthy multiparous nonlactating dairy cows (3.8 ± 0.2 yr old; 545 ± 25 kg) were used for this experiment. All procedures were performed at the department of Veterinary Sciences, University of Antwerp. The experimental protocol was approved by the Ethical Committee on Animal Experimentation (ECD 2013–15, University of Antwerp).

The experimental design of this study is shown in Figure 1. After a 6-wk acclimatization period before the start of the experiment, in which the animals were fed ad libitum (hay and concentrates), cows were subjected to 4 consecutive dietary treatments of 28 d each: (1) 1.2 × maintenance (M) or positive energy balance (PEB) without bC supplement (PEB-bC), (2) 1.2 × M with daily supplement of 2,000 mg of bC comparable to the level of bC intake at grazing

(Kawashima et al., 2010; 20 g of Rovimix β-carotene 10%, DSM Nutritional Products, Kaiseraugst, Switzerland; PEB+bC), (3) 0.6 × M with 2,000 mg of bC (NEB+bC), and (4) 0.6 × M (NEB-bC). Due to the relative long half-life of bC (approximately 5 d; Dimitrov et al., 1986; Schwedhelm et al., 2003), a 6-wk wash-out period (ad libitum feed intake) was included after the third treatment to reduce blood bC concentrations to basal levels. During the experimental period, cows were fed twice daily. Cows were individually tied up in a stable next to each other to have full control of their proper energy intake. Five times a week, during half a day, cows had a paddock at their disposal with no grass available. Rations were calculated per BW and consisted of average quality hay, chopped straw, and concentrates (15% CP, 4% crude fat, 7.5% crude ash, and 14.5% crude cellulose; Table 1). Body weight was monitored weekly.

Figure 1. In vivo experimental design. (A) Presentation of the 4 consecutive dietary treatments including a 6-wk wash-out period inserted before the last treatment. (B) Presentation of the synchronization protocol during the last 9 d of each dietary treatment. PEB = positive energy balance; NEB = negative energy balance; FF = follicular fluid; supp = supplementation; bC = β-carotene (2,000 mg/d or 20 g of Rovimix β-carotene 10%, DSM Nutritional Products, Kaiseraugst, Switzerland); PRID = progesterone intravaginal device.

To synchronize the moment of ovulation in all cows, a progesterone-releasing intravaginal device (**PRID**; 1.55 g of progesterone, CEVA, Brussels, Belgium) was used. During the last 9 d of each dietary period, cows were synchronized by placing a PRID for 7 d and a PGF_{2α} injection (Dinolytic, Pfizer, Elsenne, Belgium) on d 6 after PRID insertion (Figure 1B). Together with FF collection 36 h after PRID removal, blood was sampled.

Sampling of FF and Blood

At the end of each dietary period, 36 h after PRID removal, FF was collected (Figure 1). Dominant follicles (>8 mm) were punctured because they contain all nutrients necessary for the oocyte to be ready to ovulate. Follicular fluid was sampled using the ultrasound-guided transvaginal oocyte pick-up method as described by Leroy et al. (2004b). Briefly, the rectum was emptied and the external genitalia were carefully disinfected. All cows received an epidural injection (5 mL of Procain HCl 4%, VMD, Arendonk, Belgium) to prevent them from straining. An ultrasound device with a 7.5-MHz electronic sector transducer (Pie Medical Imaging, Maastricht, the Netherlands) and needle guidance system was inserted into the vagina. Through rectal manipulation, both ovaries were visualized and the diameter of the dominant follicle was recorded. The dominant follicle was positioned on the biopsy line, punctured, and FF was aspirated by means of a 5-mL syringe connected to the needle by a stainless steel connector to a thin silicone tube (Bols et al., 1995). Samples contaminated with blood were omitted from the study. Immediately after aspiration, the collected FF samples were cooled on melting ice until further processing (4°C). Within 3 h, FF was centrifuged for 10 min (10,000 × *g*, 4°C; Leroy et al., 2004b) and supernatant was frozen (−80°C) until further analysis on retinol, α-tocopherol (**aT**), bC, FFA, estrogen (**E2**), and progesterone (**P4**) concentrations.

Plasma and serum samples were collected from each cow on the day of FF sampling. Plasma was sampled from a jugular vein in EDTA tubes (Vacuette, 9 mL of K2E, Greiner Bio-One, Wommel, Belgium) and serum in clot activating tubes (Vacuette, 9 mL of Serum Clot Activator). After the EDTA blood tubes were gently mixed, they were immediately put on ice until further processing. The coagulated blood samples were kept at room temperature for a minimum of 1 h. Within 3 h, clot activating tubes were centrifuged for 30 min (1,400 × *g*) and EDTA tubes were centrifuged for 10 min (1,000 × *g*; 4°C) after which plasma, red blood cells (**RBC**), and serum were collected. Then, 50% (vol/vol) 50 mM phosphate-buffer (20 mM NaH₂PO₄, 30 mM Na₂HPO₄, Carl Roth, Karlsruhe, Germany) with 2 mM

EDTA was added to all RBC samples. All samples were stored at −80°C until they were analyzed for plasma retinol, aT and bC concentrations, RBC glutathione (**GSH**) concentrations, and serum biochemical parameters [total antioxidative status (**TAS**), FFA, triglycerides (**TG**), total cholesterol (**CH**), urea, IGF-1, growth hormone (**GH**), E2, and P4].

Biochemical Analyses

Within and between coefficients of variation are indicated between parentheses as percentages. Urea (1.7, 2.2), TG (1.3, 2.3), and total CH (1.1, 1.4) were enzymatically determined with photometric analysis (c16000 analyzers, Abbott Diagnostics, Santa Clara, CA). Estrogen (2.1, 2.2) and P4 (2.0, 3.8) were analyzed using a chemiluminescent particle immunoassay (i2000 analyzers, Abbott Diagnostics). Radioimmunoassay kits were used to measure IGF-1 (9.8, 10.4) and GH (4.4, 8.9) concentrations (Diasource, Louvain-La-Neuve, Belgium). Free fatty acids (2.5, 4.9) and TAS (9.0, between run variation) were measured photometrically using RX Daytona (Randox Laboratories, Crumlin, Ireland).

Glutathione Analyses

Within 2 wk after collection, RBC samples containing 50% phosphate/EDTA were thawed, immediately centrifuged (5,000 × *g*, 10 min, 4°C), and supernatant was transferred to centrifuge filter tubes (VWR, Leuven, Belgium) after which they were centrifuged again (14,000 × *g*, 20 min, 4°C). The RBC filtrate was diluted 1/320 with mobile phase A (3% (vol/vol) methanol (Fisher Scientific, Aalst, Belgium); 97% (vol/vol) HPLC-grade water; 25 mM NaH₂PO₄; 1.4 mM octanesulfonic acid; pH 2.7), and 5% (vol/vol) H₃PO₄ was added. A negative control (phosphate/EDTA buffer) was included with each analytical procedure. Samples (100 μL) were injected by a Gilson 234 autosampler with 832 sample cooler at 7°C and analyzed on a Gilson 321-H1 HPLC system (Gilson Inc., Den Haag, the Netherlands) with ESA-5600A CoulArray 8-channel electrochemical detector with Thermal Organizer (Thermo Scientific, Aalst, Belgium). Mobile phase B consisted of 50% (vol/vol) methanol (Fisher Scientific); 50% (vol/vol) HPLC-grade water; 25 mM NaH₂PO₄, and 1.4 mM octanesulfonic acid (pH 2.7). The HPLC flow rate was set at 0.7 mL/min and the gradient profile consisted of a 25-min linear gradient from 0 to 50% mobile phase B, a 5-min linear gradient from 50 to 100% mobile phase B, and 5 min isocratic at 100% mobile phase B before returning to initial conditions in 5 min ending with 5 min isocratic at 0% mobile phase

B. Cell potentials were set at 500, 580, 660, 740, 820, 900, 980, and 0 mV versus the palladium reference. A C18 reversed phase column (4 × 250 mm; 5 µm; Merck, Darmstadt, Germany) was used.

Vitamin Analyses

Plasma samples were analyzed on fat-soluble vitamins as described by Hermans et al. (2005) with minor modifications, using a validated HPLC 8-coulometric method. Briefly, fat-soluble vitamins were extracted from plasma and standards by means of n-hexane (Fisher Scientific). A negative control was included with each analytical procedure. Following centrifugation (4,000 × g, 10 min, 4°C), supernatant was collected and this extraction procedure was repeated twice. All supernatant was evaporated by vacuum centrifugation and the residue was redissolved in ethanol and analyzed on a Gilson 321-H1 HPLC system with ESA-5600A CoulArray 8-channel electrochemical detector with Thermal Organizer. During the analysis, samples were cooled at 7°C. Mobile phase A consisted of methanol (Fisher Scientific): HPLC grade water: 1 M ammonium acetate (Merck; 90:8:2; pH 4.4). Mobile phase B consisted of methanol: 1-propanol (Labscan, Samutsakorn, Thailand): 1 M ammonium acetate (78:20:2; pH 4.4). The HPLC flow rate was 0.6 mL/min and the gradient profile consisted of a 5-min constant gradient at 0% mobile phase B, a 21-min linear gradient from 0 to 80% mobile phase B, a 10-min linear gradient from 80 to 100% mobile phase B, and 14 min isocratic at 100% mobile phase B before returning to initial conditions in 5 min. Cell potentials were set at 200, 400, 500, 700, 800, -1,000, 200, and 500 mV versus palladium reference. An ODS Hypersil 150 column (3 × 150 mm; 3 µm; Thermo Scientific) was used and the system temperature was set at 37°C.

Statistical Analyses

All biochemical data are presented as means ± SEM. Linear mixed models were fitted to model the effect of the energy balance (EB) and bC supplementation on the serum and FF biochemical parameters (JMP Pro 11, SAS Institute Inc., Cary, NC). As fixed effects, we included the main effects of EB and bC supplementation as well as their interaction. If the interaction term was significant, the effect of supplementation (or of EB) on the outcome parameter differed according to the status of the other fixed factor (EB or supplementation). In that case, separate effect sizes and significance levels were calculated for each fixed factor separately. If no significant interaction between EB and supplementation was present, main effect sizes for

EB and bC supplementation were calculated as well as their significance level. To account for the relatedness between observations within the same cow, a random intercept term for cow identification number was added to the model. Pairwise correlation between plasma and FF values were expressed using the Spearman correlation coefficient. Values of $P < 0.05$ were considered significant.

RESULTS

Raw data are shown in Table 2 including P -values of the interaction term and main effects of EB and bC supplementation. Effect sizes are expressed in percentages and described below.

Animals

All 6 cows lost on average 11.5% BW (65.88 ± 4.04 kg) during both NEB periods ($P < 0.001$), with initial BW at the beginning of NEB+bC (564.75 ± 27.62 kg) and NEB-bC (581.50 ± 27.81 kg) being comparable. All follicles punctured displayed an E2/P4 ratio >1 (18.10 ± 4.65 on average), indicating the collection of FF of estrogen-dominated healthy follicles only.

Vitamin Levels in Plasma and FF in Nonlactating NEB Cows

β-Carotene. The same profile of bC concentrations can be seen in plasma and FF, implying a significant correlation between plasma and FF bC levels ($\rho = 0.645$; Figure 2A), whereby FF bC concentrations were on average 10 times lower compared with plasma levels. Despite the nonsignificant interaction between EB and bC supplementation, the effect of EB was analyzed for the supplemented and nonsupplemented groups separately. The NEB significantly reduced follicular bC concentrations by 76.2% when cows were not supplemented with bC ($P < 0.05$). No significant effect of EB was observed in plasma. On the other hand, daily bC supplementation of 2,000 mg caused a significant rise in bC concentrations in both compartments. Plasma bC concentrations increased by 332.9% ($P < 0.001$) following supplementation, whereas FF bC concentrations increased by 280.8% ($P < 0.001$), independent of the energy status.

Retinol. In contrast to bC, plasma and FF retinol (vitamin A) concentrations were in the same range. Both retinol concentrations correlated significantly ($\rho = 0.715$; Figure 2B). Moreover, plasma retinol was correlated with bC in the blood as well ($\rho = 0.678$), but no significant correlation was found between FF retinol and FF bC ($\rho = 0.194$).

Table 2. Mean ($\pm$ SEM) weight change and blood and follicular fluid (FF) parameters of 6 nonlactating dairy cows during 4 different dietary treatments¹

Item ²	PEB-bC	PEB+bC	NEB-bC	NEB+bC	P (EB $\times$ bC)	P (EB effect)	P (bC effect)
Weight change (kg)	13.80 $\pm$ 5.90	8.25 $\pm$ 5.52	-60.17 $\pm$ 6.43	-71.58 $\pm$ 4.18	NS	<0.0001	NS
Biochemical parameters							
FFA (mM)							
Serum	0.20 $\pm$ 0.03	0.22 $\pm$ 0.06	0.32 $\pm$ 0.06	0.40 $\pm$ 0.09	NS	0.0063	NS
FF	0.10 $\pm$ 0.01	0.11 $\pm$ 0.01	0.16 $\pm$ 0.07	0.15 $\pm$ 0.03	NS	NS	NS
Serum total CH (mg/dL)	123.28 $\pm$ 12.53	112.12 $\pm$ 11.78	84.88 $\pm$ 6.03	101.97 $\pm$ 10.30	0.0075	—	—
Serum TG (mg/dL)	27.33 $\pm$ 3.32	25.38 $\pm$ 3.30	22.68 $\pm$ 2.38	25.92 $\pm$ 2.03	NS	NS	NS
Serum urea (mg/dL)	15.90 $\pm$ 1.89	11.02 $\pm$ 1.24	17.12 $\pm$ 1.29	13.02 $\pm$ 1.43	NS	NS	0.0058
Serum IGF-1 (ng/mL)	156.83 $\pm$ 29.71	162.67 $\pm$ 29.00	157.67 $\pm$ 26.59	178.00 $\pm$ 33.80	NS	NS	NS
Serum GH (mIU/L)	0.62 $\pm$ 0.04	0.72 $\pm$ 0.06	0.67 $\pm$ 0.08	0.61 $\pm$ 0.04	NS	NS	NS
E2 (ng/mL)	0.07 $\pm$ 0.01	0.09 $\pm$ 0.02	0.02 $\pm$ 0.01	0.06 $\pm$ 0.03	NS	0.0445	NS
Serum	1,266.00 $\pm$ 79.11	1,285.80 $\pm$ 211.06	968.67 $\pm$ 175.42	1,094 $\pm$ 137.88	NS	NS	NS
P4 (ng/mL)							
Serum	0.26 $\pm$ 0.05	0.19 $\pm$ 0.04	0.20 $\pm$ 0.04	0.21 $\pm$ 0.05	NS	NS	NS
FF	87.88 $\pm$ 16.87	152.02 $\pm$ 46.74	44.45 $\pm$ 4.99	90.60 $\pm$ 22.17	NS	NS	0.0409
Follicular diameter (mm)	13.60 $\pm$ 1.01	20.40 $\pm$ 2.29	14.08 $\pm$ 0.44	13.15 $\pm$ 0.74	0.0063	—	—
Oxidative stress parameters							
RBC GSH (μ g/mL)	657.21 $\pm$ 49.44	733.76 $\pm$ 78.11	466.64 $\pm$ 49.91	591.47 $\pm$ 42.71	NS	<0.0001	0.0036
Serum TAS (mM)	1.21 $\pm$ 0.04	1.17 $\pm$ 0.01	1.14 $\pm$ 0.03	1.13 $\pm$ 0.02	NS	0.0077	NS
Vitamin levels							
bC (μ g/mL)							
Plasma	1.02 $\pm$ 0.37	3.04 $\pm$ 0.52	0.44 $\pm$ 0.08	3.28 $\pm$ 0.32	NS	NS	<0.0001
FF	0.21 $\pm$ 0.05	0.48 $\pm$ 0.13	0.05 $\pm$ 0.01	0.51 $\pm$ 0.07	NS	NS	<0.0001
Retinol (μ g/mL)							
Plasma	0.22 $\pm$ 0.02	0.38 $\pm$ 0.03	0.25 $\pm$ 0.02	0.35 $\pm$ 0.05	NS	NS	0.0003
FF	0.25 $\pm$ 0.04	0.40 $\pm$ 0.03	0.29 $\pm$ 0.04	0.23 $\pm$ 0.05	NS	NS	0.0275
aT (μ g/mL)							
Plasma	0.89 $\pm$ 0.11	0.81 $\pm$ 0.10	0.79 $\pm$ 0.08	0.87 $\pm$ 0.15	NS	NS	NS
FF	0.29 $\pm$ 0.05	0.23 $\pm$ 0.05	0.24 $\pm$ 0.03	0.30 $\pm$ 0.05	NS	NS	NS

¹P-values of the interaction between energy balance (EB) and β -carotene (bC) supplementation [$P(EB \times bC)$] are presented. If no significant interaction was found, P-values of the main effect of EB [$P(EB \text{ effect})$] and bC supplementation [$P(bC \text{ effect})$] are given. A P-value <0.05 is considered significant.

²Weight change is the mean difference in weight between the beginning and end of each dietary period. PEB = positive energy balance; NEB = negative energy balance; FFA = free fatty acid; CH = total cholesterol; TG = triglycerides; GH = growth hormone; E2 = estrogen; P4 = progesterone; RBC GSH = red blood cell glutathione; TAS = total antioxidant status; aT = α -tocopherol.

Plasma and FF retinol concentrations were similarly influenced by daily supplementation during NEB and PEB (no significant interaction EB $\times$ bC). Both plasma and FF retinol levels increased after daily bC supplementation by 55.3% ($P < 0.001$) and 16.7% ($P < 0.05$), respectively.

α -Tocopherol. Follicular aT concentrations were 3 to 4 times lower compared with plasma levels. No significant differences were found in plasma and FF aT concentrations with regard to the energy status or bC supplementation.

Oxidative Stress Levels in Nonlactating NEB Cows

β -Carotene supplementation raised GSH concentrations in RBC both in NEB and PEB in the same way (no significant interaction of EB $\times$ bC). A reduction in DMI significantly reduced average RBC GSH concentrations by 23.9% ($P < 0.001$) compared with PEB cows. Regardless of EB, daily bC supplementation significantly raised these RBC GSH levels by 17.9% ($P < 0.01$) compared with nonsupplemented cows. The total capacity to reduce oxidative agents in the blood (no significant interaction EB $\times$ bC), measured by the TAS in serum, was significantly reduced in NEB with 4.6% ($P < 0.01$) compared with PEB cows, but could not be significantly altered by bC supplementation (+bC versus -bC; $P > 0.05$).

Biochemical Parameters

No significant interaction was found between EB and bC (EB $\times$ bC) regarding serum and FF FFA concentrations. As such, bC supplementation did not affect serum and FF FFA concentrations. A dietary induced NEB significantly increased serum FFA concentrations by 71.4% ($P < 0.01$), but not follicular FFA concentrations, compared with PEB cows. Depending on the energy status, bC supplementation had a different effect on total serum CH (significant interaction EB $\times$ bC of $P < 0.01$). Total CH significantly decreased by 31.1% ($P < 0.01$) in NEB when not supplemented and by 9.1% ($P < 0.05$) in NEB when supplemented with bC compared with PEB cows. Total CH levels significantly increased by 20.1% ($P < 0.05$) in NEB when bC was supplemented compared with nonsupplemented NEB cows. No significant difference was found in total serum CH in PEB when supplemented with bC or not.

No significant effects of energy status or bC supplementation were found on serum TG, IGF-1, or GH concentrations. The bC supplementation significantly reduced serum urea concentrations by 27.2% ($P < 0.01$), independent of the energy status (no significant interaction of EB $\times$ bC). No significant interaction be-

tween EB and bC supplementation was found on serum and FF E2 and P4 concentrations. A reduction in DMI significantly reduced circulating serum E2 concentrations by 50.0% ($P < 0.05$), but not P4 levels, when compared with PEB cows. Moreover, daily bC supplementation did increase follicular P4 concentrations 83.3% ($P < 0.05$), but not E2 levels. Follicular diameters of the punctured dominant follicles depended on EB and supplementation (significant interaction of EB $\times$ bC). When not supplemented, follicular diameters were not significantly affected by EB. In contrast, when supplemented, NEB significantly reduced follicular diameters by 35.5% compared with PEB cows ($P < 0.05$). Whereas supplementation had no significant effect on follicular diameter in NEB, in PEB, follicular diameters were 50.0% larger with supplementation compared with diameters in nonsupplemented PEB cows ($P < 0.05$).

DISCUSSION

β -Carotene has been proposed as an important antioxidant in the metabolically stressed postpartum dairy cow. Its presence in the blood and FF is subject to a series of changes during this transition period such as the milk production level and NEB status of a cow. In this context, we showed, using a nonlactating dietary induced NEB cow model, that this NEB status was able to reduce follicular bC levels and other plasma antioxidant parameters such as TAS and RBC GSH, but not plasma bC and retinol concentrations in supplemented and nonsupplemented cows. However, after daily bC supplementation, bC and retinol concentrations drastically increased in both blood and FF compartments and RBC GSH levels could be restored to levels present in healthy PEB cows. Additionally, follicular diameter increased in PEB cows after supplementation with bC.

Evidence exists (Michal et al., 1994; Chawla and Kaur, 2004; Calderón et al., 2007) that plasma bC concentrations significantly decrease during the periparturient period in high-producing dairy cows. In our dietary induced NEB model, we observed a higher increase in plasma and FF bC in NEB when supplemented (8- to 10-fold increase) compared with the increase seen after supplementation in PEB cows (2- to 3-fold increase). Regardless of the significant reduction in follicular bC, we could not substantiate this negative effect in plasma bC in our undernourished NEB cow model. It is important to mention that, compared with the NEB status in the present study, the periparturient metabolic state in lactating dairy cows is more complex and bC can be reduced due to several factors: (1) loss of bC in colostrum and milk (Calderón et al., 2007; Kankofer and Albera, 2008), (2) low-quality forages during the dry period with lower bC concentrations in the diet

(Calderón et al., 2007), (3) health problems such as liver steatosis (resulting in disturbed bC redistribution and metabolism; Jorritsma et al., 2004), and (4) even higher systemic OS levels (Bernabucci et al., 2005; Pedernera et al., 2010). Furthermore, periparturient dairy cows have a reduced DMI and thus less bC uptake from the ration. This is an important factor that was not taken into consideration in our controlled experimental set-up because the amount of bC supplement was fixed throughout NEB and PEB treatment periods. Despite this discordance, but free of many important confounders seen in similar studies in lactating cows, our data emphasize the possibility of bC supplementation to substantially increase bC levels in plasma and FF in undernourished cows.

β -Carotene was supplemented in a dosage exceeding daily recommendations (Calsamiglia and Rodríguez, 2012), but matching the amount of total bC intake at grazing (Kawashima et al., 2010). After dietary intake, bC will be absorbed in the intestines where it can partly be metabolized to retinol (vitamin A). In the liver, bC present in chylomicrons can be metabolized or temporally stored (Yang et al., 1992). Stored bC will be released in the blood circulation as lipoproteins, of which the high-density lipoproteins (**HDL**) are the major transporter for bC in the blood, with large HDL containing more bC than small-sized HDL (Ashes et al., 1984; Schweigert et al., 1987). The basal membrane of the blood-follicle barrier allows only the passage of small HDL, explaining the observed lower follicular bC concentrations compared with plasma (Perret et al., 1985; Zamah et al., 2009). Additionally, granulosa cells of the follicular wall can actively take up and convert bC to its nonantioxidant metabolite retinol (Schweigert et al., 1988; Schweigert and Zucker, 1988; Brown et al., 2003). This explains the lower follicular bC concentrations compared with plasma and might contribute to the observed high intra-follicular retinol concentrations (in contrast to bC, follicular retinol levels were very much like plasma levels). This can also be a result of a different transport mechanism of retinol (retinol binding proteins), compared with bC (Noy, 2000), and may also indicate why no correlation was found between bC and retinol in the follicle. If not selectively taken up by follicle cells, retinol passively diffuses into FF (Ikeda et al., 2005). The average physiological plasma bC and retinol concentrations are in line with previous reports of plasma bC and retinol levels in dairy cows (Chew et al., 1984; Haliloglu et al., 2002; LeBlanc et al., 2004), but bC levels are deficient based on current guidelines. Hence, it would be recommended to supplement these cows with bC (Schweigert and Immig, 2007; Calsamiglia and Rodríguez, 2012).

Bernabucci et al. (2005) showed that a severe decrease in body condition in periparturient cows is associated with higher levels of OS. This relation between energy status and systemic OS was indicated in our study by the significant lower erythrocyte levels of GSH and TAS in NEB compared with PEB cows. Furthermore, daily bC supplementation could alleviate these high OS levels in NEB cows by significantly increasing RBC GSH concentrations, but not TAS, to PEB levels again. Care needs to be taken when interpreting TAS as a proxy for the cow's OS status, because it only shows a general image. Depletion of one antioxidant can be masked by the rise of another antioxidant or a higher production of overall reactive oxygen species can be counteracted by the subsequent elevated action of naturally present antioxidants (Hermans et al., 2007).

Reports on the effect of nutrient restriction and NEB in dairy cows on follicular diameter are contradictory (Lucy et al., 1991; Mackey et al., 1999; Comin et al., 2002; Diskin et al., 2003). Most researchers report a restricted follicular growth during a period of reduced dietary intake, which could not be confirmed in our experiment. In contrast, we observed an increased follicular diameter in supplemented PEB animals compared with supplemented NEB and nonsupplemented PEB cows. Except for the findings of Kawashima et al. (2009), who described a positive relation between the start of postpartum ovarian activity in cows with higher circulating bC levels, evidence is lacking of a direct influence of bC on follicular growth. The finding of Schweigert and Zucker (1988) is noteworthy, showing that follicular retinol concentrations are higher in large good quality follicles, whereas follicular bC is not influenced by follicle size or quality, emphasizing the importance of retinol in follicle development. Retinol has been identified as a growth-promoting factor (Goldblith and Joslyn, 1964). As such, we assume that the significant higher retinol levels in FF after daily bC supplementation can stimulate follicular growth through granulosa cell proliferation (Schweigert and Zucker, 1988).

Follicular fluid of growing dominant pre-ovulatory follicles was sampled, which was supported by the much higher follicular E2 concentrations than P4 levels at the moment of FF sampling. The significant negative effect of dietary restriction, and thus NEB, on circulating E2 levels confirmed the findings of Kendrick et al. (1999) and Comin et al. (2002). Evidence exists that antioxidant vitamins, such as bC, support steroid synthesis (Young et al., 1995; Haliloglu et al., 2002; Arellano-Rodríguez et al., 2009). Besides bC, retinol might have a steroidogenic effect as well because it has been shown that vitamin A deficiency is characterized by a reduced steroid hormone production (Ganguly et

al., 1971). In line with this, follicular P4 significantly increased with daily bC supplementation. Young et al. (1995) previously showed that bC has an antioxidant protective effect on the cholesterol side-chain cleavage enzyme cytochrome P450, stimulating the production of pregnenolone, a direct precursor of P4. Moreover, P4 plays a pivotal role in follicle dominance, oocyte ovulation, and oocyte developmental competence (for review see Fair and Lonergan, 2012). Additionally, based on previous in vitro research (Ikeda et al., 2005; Shelly et al., 2008; Rooke et al., 2012) and the evidence that bC levels in the micro-environment of the oocyte can substantially rise regardless of the energy status of the cow, we can now speculate that these follicular bC can alleviate FFA-induced OS in follicle cells and oocytes.

In conclusion, a dietary induced NEB lowers follicular bC concentrations and increases systemic OS levels, but does not negatively alter plasma bC and plasma and follicular retinol levels. However, daily supplementation with 2,000 mg of bC can significantly increase bC and retinol concentrations in the environment of the oocyte, regardless of the energy status of the cow. Consequently, bC can exert its antioxidative effects in the FF, whereas retinol may favor follicular development. These findings stress the potential of bC supplementation to positively alter the micro-environment of the oocyte, which may have a positive influence on oocyte quality in the presence of maternal metabolic stress. This can be taken into account when planning future in vivo and in vitro experiments to investigate direct effects of bC on metabolically compromised oocytes. This study is a first important step in designing management strategies to feed for optimal oocyte quality, in an attempt to tackle the subfertility problem in the modern dairy industry.

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