Efecto de la Suplementación Parenteral con Minerales y Vitaminas en los Biomarcadores de Estrés Oxidativo y el Metabolismo Hepático de Ácidos Grasos en Vacas Lecheras durante el Período de Transición

En el presente trabajo, nuestro objetivo fue estudiar los efectos de la suplementación parenteral de vitaminas y minerales en el metabolismo hepático de ácidos grasos, así como en los biomarcadores de estrés oxidativo en muestras biológicas de vacas en transición. El grupo suplementado (SG, n = 11) recibió una inyección subcutánea de 5 mL de palmitato de vitamina A 35 mg/mL, acetato de vitamina E 50 mg/mL, además de otra inyección de 5 mL de edetato de cobre 10 mg/mL, edetato de zinc 40 mg/mL, edetato de manganeso 10 mg/mL y selenito de sodio 5 mg/mL en los días -60, -30 y 7 (± 3) respecto al parto. El grupo control (CG, n = 11) recibió dos inyecciones subcutáneas de 5 mL de cloruro de sodio 9 mg/mL en los mismos momentos que el SG. Se tomaron muestras de sangre, orina y biopsias de hígado 21 (± 3) días antes de la fecha esperada de parto, y 7 y 21 (± 3) días después del parto. Los resultados revelaron que los animales suplementados tenían una mayor actividad de glutation peroxidasa (GSH-Px), una concentración más baja y más alta de 3-nitrotirosina (3-NT) en el hígado y plasma, respectivamente, una mayor expresión de la enzima de beta-oxidación mitocondrial carnitina palmitoiltransferasa 1 en el hígado y un menor contenido de triacilglicerol hepático, lo que refleja los parámetros de función hepática plasmática. No se encontraron diferencias entre los grupos en la actividad de superóxido dismutasa, las concentraciones de MDA, la abundancia de proteínas acil-CoA oxidasa 1 peroxisomal, la diacilglicerol O-aciltransferasa 1 y el receptor alfa activado por el proliferador de peroxisomas. Estos resultados sugieren que la suplementación de vitaminas y minerales proporcionada a las vacas lecheras tuvo un efecto beneficioso en la actividad de GSH-Px, la concentración hepática de 3-NT y en la adaptación metabólica durante el período periparto.

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RESEARCH

Effect of Parenteral Supplementation of Minerals and Vitamins on Oxidative Stress Biomarkers and Hepatic Fatty Acid Metabolism in Dairy Cows During the Transition Period

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Abstract

In the present work we aimed to study the effects of parenteral vitamin and mineral supplementation on hepatic fatty acid metabolism as well as on the oxidative stress biomarkers in biological samples of transition cows. The supplemented group (SG, $n = 11$) received a subcutaneous injection of 5 mL of vitamin A palmitate 35 mg/mL, vitamin E acetate 50 mg/mL plus other injection of 5 mL of copper edetate 10 mg/mL, zinc edetate 40 mg/mL, manganese edetate 10 mg/mL, and sodium selenite 5 mg/mL on days − 60, − 30, and 7 (± 3) relative to calving. The control group (CG, $n = 11$) received two subcutaneous injections of 5 mL of 9 mg/mL sodium chloride at the same times of the SG. Blood, urine, and liver biopsies were sampled 21 (± 3) days before the expected calving date and 7 and 21 (± 3) days after calving. Results revealed that supplemented animals had higher glutation peroxidase (GSH-Px) activity, lower and higher concentration of 3-nitrotyrosine (3-NT) in the liver and plasma, respectively, higher expression of the mitochondrial beta-oxidation enzyme carnitine palmitoyltransferase 1 in the liver, and lower content of hepatic triacylglycerol, mirroring plasma liver function parameters. No differences between groups were found in the superoxide dismutase activity, MDA concentrations, the protein abundance of peroxisomal acyl-CoA oxidase 1, diacylglycerol O-acyltransferase 1, and peroxisome proliferator-activated receptor alpha. These results suggest that the vitamin and mineral supplementation provided to dairy cows had a beneficial effect on GSH-Px activity, hepatic 3-NT concentration, and on the metabolic adaptation during the peripartum period.

Keywords Liver fatty acid metabolism · Vitamin and mineral supplementation · Oxidative stress biomarkers · Dairy cattle · Transition period

Introduction

The postpartum period of dairy cows is characterized by an increase in the animal energy requirements, which compromises the dry matter intake (DMI). Consequently, animals develop a characteristic negative energy balance (NEB) with increased lipolysis of adipose tissue, which leads to a greater flow of plasma non-esterified fatty acids (NEFA) toward the liver, where they can finally be metabolized [1]. When the liver capacity to metabolize fatty acids is exceeded, metabolic disorders such as fatty liver can occur [2].

The drastic changes in nutritional and energy requirements also exacerbate the production of reactive oxygen and nitrogen species (RONS), which induce oxidative stress (OS) [3]. Given the high instability and low half-life of RONS, their quantification is complex; however, as they are highly reactive molecules that can interact with a variety of biomolecules, a wide range of possible biomarkers have emerged to assess oxidative damage [4, 5]. One of these interactions is the nitration of protein tyrosine by peroxynitrites and other RONS, which leads to the stable end product 3-nitrotyrosine (3-NT)

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[6, 7]. Other OS biomarker is malondialdehyde (MDA), the principal and most studied product of polyunsaturated fatty acid peroxidation, which have been studied in different species, including healthy cows [8–11]. The imbalance caused by the increased production of RONS together with a lower availability of antioxidant defenses could increase the susceptibility of dairy cows to develop postpartum diseases [12, 13].

Previous studies in humans and rats have shown a relationship between OS levels and the degree of obesity and liver lipidosis [14]. In dairy cows, a previous study has also found a relationship between the body condition score (BCS) and parameters of OS in plasma [15]. Furthermore, it has been shown that fatty acid oxidases lead to the synthesis of H_2O_2, which could contribute to the oxidative phenomenon [16, 17]. Thus, in the last decades, there has been a growing interest in evaluating whether treatments with antioxidants, omega-3 polyunsaturated fatty acids, and conjugated linoleic acid can prevent OS [18, 19]. Some antioxidants are micronutrients, such as vitamins A and E, and the minerals which are a structural part of antioxidant enzymes [20, 21]. In this sense, superoxide dismutase (SOD) is a copper-, manganese-, and zinc-dependent enzyme and GSH-Px is a selenoprotein [22]. In this regard, human studies have shown that patients with non-alcoholic fatty liver disease had a decrease in the activities of SOD and GSH-Px enzymes [23]. Cortinhas et al. [24] and Machado et al. [25] have shown beneficial effects of parenteral supplementation with these minerals on cell count scores, clinical mastitis, and incidence of endometritis in dairy cattle. In addition, Teixeira et al. [26] observed that an injectable trace mineral supplement in dairy calves led to increased neutrophil function. Moreover, a recent revision has mentioned that selenium supplementation ameliorates the antioxidant system with a reduction of the incidences of metabolic diseases such as fatty liver and ketosis in periparturient dairy cattle [27].

Based on all the above, the hypothesis of the present study was that vitamin and mineral supplementation can have beneficial effects on OS biomarkers in different biological matrices and on hepatic fatty acid metabolism in dairy cattle during the transition period. Thus, to test our hypothesis, we analyzed the concentrations of free 3-NT and MDA in different biological matrices, antioxidant status biomarker, the liver content of triacylglycerol, and the abundance of key proteins involved in fatty acid metabolism, such as carnitine palmitoyltransferase 1 (CPT-1), peroxisomal acyl-CoA oxidase 1 (ACOX-1), diacylglycerol O-acyltransferase 1 (DGAT-1), and the peroxisome proliferator-activated receptor alpha (PPAR-α), in dairy cows supplemented with vitamins and minerals relevant to the antioxidant system during the transition period.

Materials and Methods

Animals

The present study was carried out according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (4° Ed. American Dairy Science Association®, American Society of Animal Science, and Poultry Science Association. 2020) and approved by the Ethics Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Santa Fe, Argentina (protocol number 292/16).

Twenty-two Holstein cows were from a commercial grazing dairy farm with a grass-based feeding system. The animals were close to entering their second to fourth lactation, drying off 60 days before the expected calving date with antibiotic dry cow therapy. The farm is localized in Hipatia (31°07′S, 61°03′W), Santa Fe province, Argentina, and, at the time of the study, had 500 lactating cows that milked twice daily in one of the two parlors of a double-24 parallel parlor and averaged 26 kg of milk per cow per day during the study period. Sixty days (± 3) before the expecting calving date, the animals were divided into two groups: the supplemented group (SG; $n = 11$), which received one injection of 5 mL of the vitamin supplement ADAPTADOR® Vit (Biogenesis Bagó, Buenos Aires, Argentina; vitamin A palmitate 35 mg/mL and vitamin E acetate 50 mg/mL) plus other injection of 5 mL of the mineral supplement ADAPTADOR® Min (Biogenesis Bagó; copper edetate 10 mg/mL, zinc edetate 40 mg/mL, manganese edetate 10 mg/mL, and sodium selenite 5 mg/mL) on days − 60, − 30, and 7 (± 3) relative to calving; and the control group (CG; $n = 11$), which received two injections of 5 mL of 9 mg/mL sodium chloride at the same times as those indicated for the SG.

Samples of major feed ingredients were taken monthly and sent to an external laboratory for analysis (Rock River Laboratory, Santa Fe, Argentina). The diet was formulated by using the NRC 2001 software and the composition is summarized in Table 1. A diet based on alfalfa and ryegrass was administered ad libitum to the animals in the postpartum period and other components were available through a mixed diet. Herbage DMI was estimated from pre- and post-grazing herbage mass per m2, multiplied by the assigned area/cow. Before and during the experiments, the animals were clinically checked and showed no evidence of disorders such as retained placenta, clinical milk fever, mastitis, metritis, clinical ketosis, displaced abomasum, lameness, and/or clinical gastrointestinal disorders. Three times a week, a veterinary specialist monitored the health status of cows based on clinical signs of disease and by observing external symptoms such as rectal temperature, color and consistency

Table 1 Ingredients and chemical composition of the diet for prepartum and postpartum cows of the experimental herds

Ingredient (% DM)	Prepartum	Postpartum
Corn silage	19.52	20.22
Cracked corn grain	7.18	20.95
Soybean silage		9.24
Soybean meal	7.18	4.34
Wheat grain		6.25
Alfalfa grazing		16.37
Ryegrass grazing		21.67
Sunflower meal	21.50	
Wheat straw	43.01	
Mineral and vitamin pack[1]	1.60	0.96
Estimated DMI (kg/d)	12.6	20.8
Chemical		
CP (% DM)	13.8	18.2
NDF (% DM)	50.8	31.7
ADF (% DM)	39.4	19.2
NFC (% DM)	29.4	42.3
NEl (Mcal/kg)	1.40	1.63

[1]The prepartum pack contained Mg 15%, Cr 1%, Zn 1600 ppm, Mn 650 ppm, Cu 750 ppm, I 25 ppm, Se 2 ppm, Co 19 ppm, Vit A 350,000 IU/kg, Vit D 60,000 IU/kg, Vit E 6500 IU/kg, rumen-protected choline 25%, and rumen-protected methionine 8%, whereas the postpartum pack contained Ca 24%, Mg 8%, Na 15%, Cl 10%, monensin 4000 ppm, Zn 3400 ppm, Mn 1000 ppm, Cu 800 ppm, I 46 ppm, Se 25 ppm, Co 8 ppm, Vit A 300,000 IU/kg, Vit D 80,000 IU/kg and Vit E 1000 IU/kg

%DM, dry matter basis; *ADF*, acid detergent fiber; *NFC*, non-fiber carbohydrate; *DMI*, dry matter intake

of vaginal discharges, body condition score, gait, general status, and alertness. The veterinary specialist also performed auscultation and percussion of the abdomen. Only healthy animals were included in the study. After parturition, cows were milked twice a day and milk production was recorded monthly up to 120 days in milk with milk meters (Waikato Milking Systems, Hamilton, New Zealand).

Sample Collection

Blood, urine, and liver samples were simultaneously collected 21 (± 3) days before the expecting calving date and 7 (± 3) and 21 (± 3) days after calving. Also, at the same time, the BCS of cows was recorded based on a scale described by Edmonson et al. (1989).

Cows were individually restrained by using a manual head gate and blood was sampled from the *jugular vein* and collected into ethylenediaminetetraacetic acid (EDTA), heparin, and non-anticoagulant tubes. To separate the plasma and serum, tubes were centrifuged for 10 min at 2000 × *g*. Urine samples were collected in sterile bottles by stimulating the area below the vulva. Plasma, serum, and urine samples were stored at – 80 °C.

Finally, approximately 500 mg of liver tissue was sampled via puncture biopsy as previously described [28] and fractionated in two tubes: one of them without buffer and the other containing an antioxidant buffer that consisted of 100 µmol/L diethylenetriaminepentaacetic acid, 50 µmol/L butylated hydroxytoluene in 1% (v/v) ethanol, and 10 mmol/L 3-amino-1,2,4-triazole in 50 mmol/L sodium phosphate buffer, pH 7.4 [29]. Liver samples were snap frozen in liquid nitrogen and subsequently stored at – 80 °C.

Assessment of Plasma Metabolites and Liver Triacylglycerol (TAG)

The concentration of β-hydroxybutyrate acid (BHBA) was determined using a handheld device (Precision Xtra meter, Abbott Diabetes Care Inc., Alameda, CA, USA) [28, 30]. Plasma concentrations of NEFA were determined using the commercial colorimetric kit number FA115, from RANDOX Laboratories LTD (UK), whereas those of glucose, albumin, total bilirubin, cholesterol, aspartate transaminase (AST), and gamma-glutamyl transpeptidase (GGT) were determined enzymatically using commercial kits from WIENER Lab (Rosario, Argentina) by using an ultra-fast UV/Vis spectrometer SPECTROstar Nano (BMG LABTECH GmbH, Ortenberg, Germany) [2]. Liver TAG content was measured as previously [28]. Briefly, total lipids were extracted from liver homogenates with a mixture of chloroform and methanol (2:1 v/v) according to the procedure of Folch et al. [31], filtering and washing the extract with suitable solutions of salts and solvents. Finally, after removing the phospholipids, the liver TAG content was measured enzymatically using a commercial kit (WIENER Lab, Rosario, Argentina).

Antioxidant Status Biomarkers

Whole blood with heparin was used to measure SOD and GSH-Px activities. Following the manufacturer's instructions, red blood cell lysates were obtained and SOD activities were measured by using a RANSOD kit (Randox Laboratories Ltd., Crumlin, UK) [10]. The GSH-Px activity was determined by using a commercial kit (RANSEL, Randox Laboratories, Crumlin, UK) and was reported in units per gram of hemoglobin. In addition, serum samples were analyzed for total antioxidant status by using a commercial colorimetric assay kit (Randox Laboratories Ltd., Crumlin, UK).

Determination of Free 3-NT and MDA in Biological Samples

Free 3-NT and MDA were analyzed in plasma, urine, and liver samples. Regarding the 3-NT biomarker, the analytical technique developed and validated in Barcarolo et al. [32] was followed for its quantification in

the matrices. To determine MDA concentrations, we have previously developed and validated the analytical methodology [10].

Determination of Protein Abundance

Approximately 50 mg of frozen liver sample was homogenized in a lysis buffer containing 1% v/v octylphenyl-polyethylene glycol (IGEPAL CA630), 50 mmol/L sodium fluoride, 1 mmol/L EDTA, 0.1% w/v sodium dodecyl sulfate (SDS), 0.5% w/v sodium desoxycholate, 0.1 mol/L PBS, and a protease and phosphatase inhibitor cocktail (all from Sigma-Aldrich Corp., St. Louis, MO, USA). Subsequently, the tissue lysates were centrifuged at 12,000 × *g* for 20 min and the supernatant was separated and stored at − 80 °C. Protein concentrations were quantified using the Lowry method with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis was performed as previously described [28]. The samples (40 µg of protein/lane) were denatured by boiling for 5 min in a Laemmli sample buffer and resolved on 10% SDS-PAGE. Then, the resolved proteins were transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). The Western blot assay was linear from 20 to 80 µg of protein and, at increasing exposure times, a standard sample from a pool of six animals was blotted on each membrane to adjust band values from different membranes.

The membranes were blocked for 1 h with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.05% (v/v) Tween-20 (Sigma-Aldrich Corp.) at room temperature and then incubated overnight at 4 °C with the specific primary antibodies (Table 2). After washing, membranes were treated for 1.5 h at room temperature with the corresponding mouse anti-rabbit IgG peroxidase-conjugated antibody (Table 2), followed by a chemiluminescence detection kit (ECL Prime Western Blotting Detection 247 Reagent, GE Healthcare) on hyperfilm-ECL film (GE Healthcare). Vinculin was used as loading control using the same protocol described above. The intensity of the bands was quantified using the IMAGE PRO-PLUS 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA).

Statistical Analysis

The size of sample was calculated a priori using the G*Power software (version 3.1.9.4; Faul et al. [33]) according to the following: measurements repeated within the interaction analysis, power of 0.8, effect size of 0.4, significance level of 0.05, correlation between repeated measures of 0.5, and correction of non-sphericity of 0.5. According to the sample size calculation, twenty animals were required. The data were analyzed using the statistical software package SPSS 25.0 for WINDOWS (SPSS Inc., Chicago, IL, USA). All analyses were performed considering the animal as the experimental unit. The distribution of data was tested for normality by using the Kolmogorov–Smirnov test. Repeated-measures analysis was performed using the generalized linear model (GLM) approach with log link function for variables with non-normal distribution. For outcome variables with normal distribution, a GLM with a linear link function was used. The first-order autoregressive covariance structure was used to assess repeated measures. Akaike information criterion between first-order autoregressive, compound symmetry and unstructured structures was considered for the

Table 2 Antibodies and conditions used for Western blot assays

Antibodies	*Type*	*Suppliers*	*Dilution*	*Protein/lane*
	Primary antibodies			
PPAR-alpha	Rabbit polyclonal H-98: sc-9000	Santa Cruz Biotechnology, Inc., CA, USA	1/500	40 µg
CPT-1-L	Rabbit polyclonal H-95:sc-20669	Santa Cruz Biotechnology, Inc., CA, USA	1/3000	40 µg
ACOX-1	Rabbit polyclonal H-140: sc-98499	Santa Cruz Biotechnology. Inc., CA, USA	1/6000	40 µg
DGAT-1	Rabbit polyclonal ab54037	Abcam, Cambridge, UK	1/7500	40 µg
Vinculin	Mouse monoclonal V284:MCA465GA	Bio-Rad Laboratories, Inc., CA, USA	1/7500	
	Secondary antibodies			
Mouse anti-rabbit IgG	Polyclonal sc-2357	Santa Cruz Biotechnology. Inc., CA, USA	1/10,000	
Mouse IgGκ binding protein	Binding protein: sc-516102	Santa Cruz Biotechnology. Inc., CA, USA	1/10,000	

selection of this covariance structure. The model consisted of supplementation (S), time (T), and S × T as fixed effects. When the S × T interaction was significant, differences between treatments at each time point were tested for significance with a GLM. The Bonferroni correction method was used for correction of multiple comparisons. A value of $P < 0.05$ was considered significant. The results are expressed as mean ± SEM.

Results

Milk Yield and BCS

The cumulative milk production at 120 days in milk tended to be higher for the SG (4631.91 ± 213.52 L) than for the CG (4163.73 ± 191.94 L) ($P = 0.10$). The BCS decreased throughout the period evaluated ($P < 0.05$), but without differences between groups or interaction effect ($P < 0.05$) (Table 3).

Plasma Parameters and Liver TAG Content

The concentrations of NEFA and BHBA were higher in the postpartum period ($P < 0.05$) but without differences between groups or interaction effect ($P > 0.05$). Glucose concentration was higher in the SG than in the CG ($P < 0.05$), without differences by time or interaction effect ($P > 0.05$).

The liver TAG content increased during the postpartum, with a peak on day 7 ($P < 0.05$), and was lower in the SG than in the CG ($P < 0.05$). No interaction effect was observed ($P > 0.05$).

Regarding liver function biomarkers, albumin concentration was higher in the SG than in the CG ($P < 0.05$), and an interaction effect was observed: albumin concentration was higher in the SG on days 21 prepartum and 7 postpartum ($P < 0.05$). No difference by time was observed ($P < 0.05$). Cholesterol concentration was higher on day 21 after calving ($P < 0.05$), without differences between groups or interaction effect ($P > 0.05$). The activity of AST was higher after calving, with a peak on day 7 ($P < 0.05$), but no differences between groups were observed ($P > 0.05$). An interaction

Table 3 BCS, concentrations of NEFA, BHBA, glucose, albumin, bilirubin, cholesterol, and AST, and GGT activity in plasma of dairy cattle

		Prepartum days	*Postpartum days*				
		−21	7	21	S	T	S × T
BCS	CG	3.16 ± 0.08	2.75 ± 0.11	2.66 ± 0.06	0.42	< 0.01	0.23
	SG	3.18 ± 0.07	2.93 ± 0.07	2.66 ± 0.07			
NEFA	CG	0.33 ± 0.05	0.90 ± 0.16	0.61 ± 0.10	0.93	< 0.01	0.30
(mmol/L)	SG	0.25 ± 0.05	0.91 ± 0.11	0.78 ± 0.11			
BHBA	CG	0.27 ± 0.05	0.88 ± 0.09	0.75 ± 0.09	0.53	< 0.01	0.63
(mmol/L)	SG	0.26 ± 0.02	0.85 ± 0.12	0.63 ± 0.06			
Glucose	CG	50.93 ± 4.77	49.49 ± 5.46	39.82 ± 4.61	< 0.01	0.24	0.25
(mg/dL)	SG	52.81 ± 3.81	63.49 ± 7.93	56.61 ± 4.47			
Albumin	CG	2.91 ± 0.21 a	3.10 ± 0.15 a	3.48 ± 0.16	< 0.01	0.37	0.02
(g/dL)	SG	3.57 ± 0.17 b	3.89 ± 0.22 b	3.49 ± 0.14			
Cholesterol	CG	77.96 ± 6.04	84.85 ± 5.88	131.51 ± 9.42	0.20	< 0.01	0.36
(mg/dL)	SG	70.20 ± 5.07	71.15 ± 5.35	123.51 ± 8.60			
Bilirubin	CG	0.79 ± 0.12	0.92 ± 0.06	0.90 ± 0.11	0.57	0.30	0.11
(mg/dL)	SG	0.93 ± 0.14	0.87 ± 0.08	0.68 ± 0.05			
AST	CG	54.68 ± 6.00	93.96 ± 5.66	72.08 ± 7.22	0.43	< 0.01	< 0.01
(IU/L)	SG	71.21 ± 9.64	80.77 ± 5.45	79.90 ± 5.19			
GGT	CG	17.67 ± 2.58	21.22 ± 3.20	24.69 ± 3.18	0.37	0.12	0.68
(IU/L)	SG	15.73 ± 2.24	20.50 ± 1.94	20.25 ± 3.07			
Liver TAG	CG	10.44 ± 2.03	26.68 ± 4.94	19.52 ± 4.18	0.03	< 0.01	0.14
(mg/g WT)	SG	9.54 ± 1.35	13.58 ± 1.61	12.80 ± 1.61			

Animals were grouped as supplemented cows (SG; $n = 11$) and control cows (CG; $n = 11$) at 21 days prepartum, and at 7 and 21 days postpartum. Values are expressed as mean ± SEM. The statistical effects of S, T, and S × T are indicated. [a–c] S × T ($P \leq 0.05$) supplementation effect at a given week. *BCS*, body condition score; *NEFA*, non-esterified fatty acids; *BHBA*, beta-hydroxybutyric acid; *AST*, aspartate transaminase; *GGT*, gamma-glutamyl transpeptidase; *ALP*, alkaline phosphatase; *Liver TAG (mg/g WT)*, liver triacylglycerol content (mg/g wet tissue); *S*, supplementation; *T*, time; *S × T*, interaction effect. Supplementation was based on vitamin A palmitate 35 mg/mL and vitamin E acetate 50 mg/mL, plus copper edetate 10 mg/mL, zinc edetate 40 mg/mL, manganese edetate 10 mg/mL, and sodium selenite 5 mg/mL

effect was recorded ($P < 0.05$), but no differences between groups at any time were observed ($P > 0.05$). Regarding bilirubin and GGT activity, no differences by time, groups, or interaction effect were observed ($P > 0.05$) (Table 3).

Antioxidant Status Biomarkers

The GSH-Px activity was higher in the SG than in the CG ($P < 0.05$), no differences by time were observed CG ($P < 0.05$). An interaction effect was recorded, but the activities in all the times were higher in the SG than in the CG ($P < 0.05$). SOD activity was lower on day 7 pospartum than on day 21 prepartum ($P < 0.05$). No differences between groups or interaction effect were observed ($P > 0.05$). Finally, no differences by time, groups, or interaction effect were observed for TAS ($P > 0.05$) (Table 4).

Free 3-NT and MDA Concentration in Plasma, Urine, and Liver Tissue

Plasma 3-NT concentration was lower on day 21 postpartum than during the prepartum and higher in the SG than in the CG ($P < 0.05$). No interaction effect was observed ($P > 0.05$). In urine, no differences by time, groups, or interaction effect were observed ($P > 0.05$). Finally, in liver tissue, 3-NT concentration was lower in the SG than in the CG ($P < 0.05$), without differences by time or interaction effect ($P > 0.05$). No differences between groups or by time were observed for plasma MDA concentration ($P > 0.05$). An interaction effect was observed ($P < 0.05$) but without differences among supplementation at a given week ($P > 0.05$). Regarding urine MDA, the concentration was higher on day 21 postpartum than on day 21 prepartum ($P < 0.05$). No differences between groups or interaction effect were observed ($P > 0.05$). Finally, liver MDA concentration was higher on day 21 postpartum than the other days evaluated ($P < 0.05$). No differences between groups or interaction effect were observed ($P > 0.05$) (Fig. 1).

Protein Abundance

The protein abundance of CPT-1 was lower on day 21 postpartum than on day 21 prepartum ($P < 0.05$). Also, the abundance of this protein was higher in the SG than in the CG ($P < 0.05$), and no interaction effect was observed ($P > 0.05$). Concerning ACOX-1 and DGAT-1, no differences by time, groups, or interaction effect were observed ($P > 0.05$). On the other hand, the protein abundance of PPARα was higher on day 7 postpartum than during the prepartum ($P < 0.05$), but no differences between groups or interaction effect were observed ($P > 0.05$) (Fig. 2).

Discussion

Several studies have evaluated the effects of different nutritional and pharmaceutic treatments on the hepatic metabolism of dairy cattle to improve milk production and/or the cow's health status [34–36]. A review article by Abuelo et al. [17] has suggested that the usual systems used to calculate the nutrient requirements of dairy cattle underestimate the requirements of vitamins and minerals and demonstrated the benefits of supplementation (slightly above the usual levels) in animal health and performance. Therefore, in this study, we aimed to evaluate the possible effects of the parenteral administration of a mineral-vitamin supplement on the metabolism of hepatic fatty acids, a critical process during the transition period of dairy cow. In addition, in this metabolic context, the concentrations of 3-NT and MDA as possible biomarkers for OS were analyzed by mass spectrometry in three biological matrices.

Table 4 GSH-Px and SOD activity in blood and serum TAS of dairy cattle

		Prepartum days	*Postpartum days*				
		−21	7	21	S	T	S×T
GSH-Px	CG	266.5 ± 23.0[a]	226.4 ± 19.1[a]	232.9 ± 25.5[a]	< 0.01	0.22	0.02
(IU/g Hg)	SG	371.9 ± 26.7[b]	393.8 ± 19.4[b]	455.5 ± 20.7[b]			
SOD	CG	322.4 ± 34.5	266.4 ± 26.0	297.1 ± 29.7	0.22	0.02	0.59
(IU/mL)	SG	389.6 ± 36.3	307.1 ± 32.5	303.6 ± 25.3			
TAS	CG	0.564 ± 0.039	0.553 ± 0.036	0.519 ± 0.061	0.20	0.29	0.32
(mmol/L)	SG	0.513 ± 0.015	0.468 ± 0.021	0.551 ± 0.034			

Animals were grouped as supplemented cows (SG; $n = 11$) and control cows (CG; $n = 11$) at 21 days prepartum, and at 7 and 21 days postpartum. Values are expressed as mean ± SEM. The statistical effects of S, T, and S × T are indicated. [a–c] S × T ($P \leq 0.05$) supplementation effect at a given week. *GSH-Px*, glutathione peroxidase; *SOD*, superoxide dismutase; *TAS*, total antioxidant status; *S*, supplementation; *T*, time; *S×T*, interaction effect. Supplementation was based on vitamin A palmitate 35 mg/mL and vitamin E acetate 50 mg/mL, plus copper edetate 10 mg/mL, zinc edetate 40 mg/mL, manganese edetate 10 mg/mL, and sodium selenite 5 mg/mL

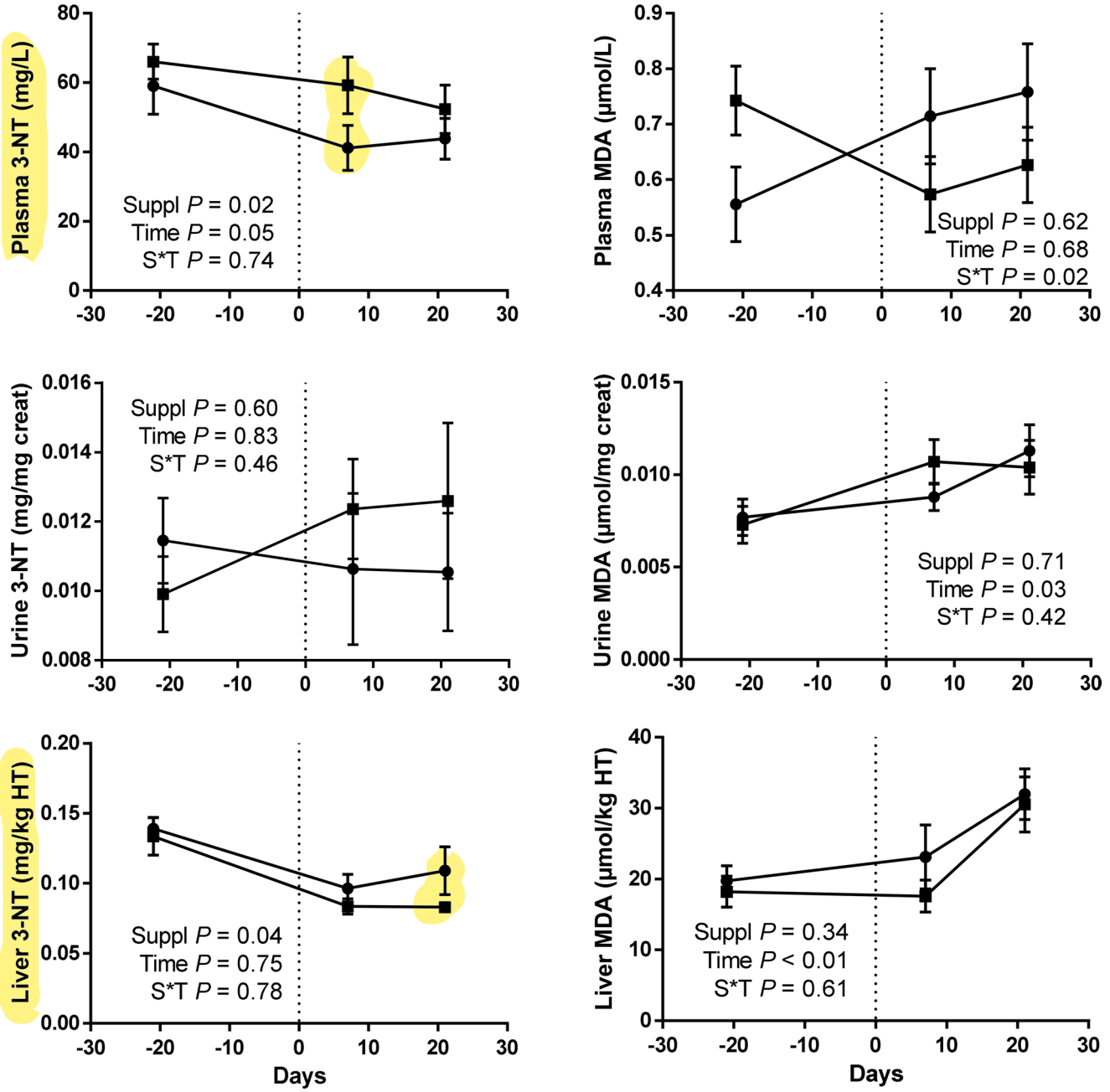

Fig. 1 Concentration of free 3-nitrotyrosine (3-NT) and malondialdehyde (MDA) in plasma, urine, and liver tissue of dairy cattle. Animals were grouped as supplemented cows (SG; $n=11$; black squares) and control cows (CG; $n=11$; black circles) at 21 days prepartum, and at 7 and 21 days postpartum. Values are expressed as mean ± SEM. The statistical effects of supplementation (Suppl), time, and supplementation × time (S × T) are indicated. [a–c] S × T ($P \leq 0.05$) supplementation effect at a given week. 3-NT, 3-nitrotyrosine; MDA, malondialdehyde; HT, humid tissue; creat, creatinine

Biochemical Parameters and Liver TAG Content

The NEB has been described as a factor predisposing to metabolic and infectious diseases [37, 38]. Since the NEB can be evaluated by the BCS, here we assessed the BCS and observed no differences between groups. Also, the prepartum values of BCS and the postpartum loss of BCS recorded are in agreement with previous recommendations [39]. In concordance with the BCS observed in animals of our study, the concentrations of NEFA and BHBA, which are NEB parameters, showed no differences between groups and were lower than the reference concentrations recorded in animals with increased risk of disease and decreased productive and reproductive performance [40].

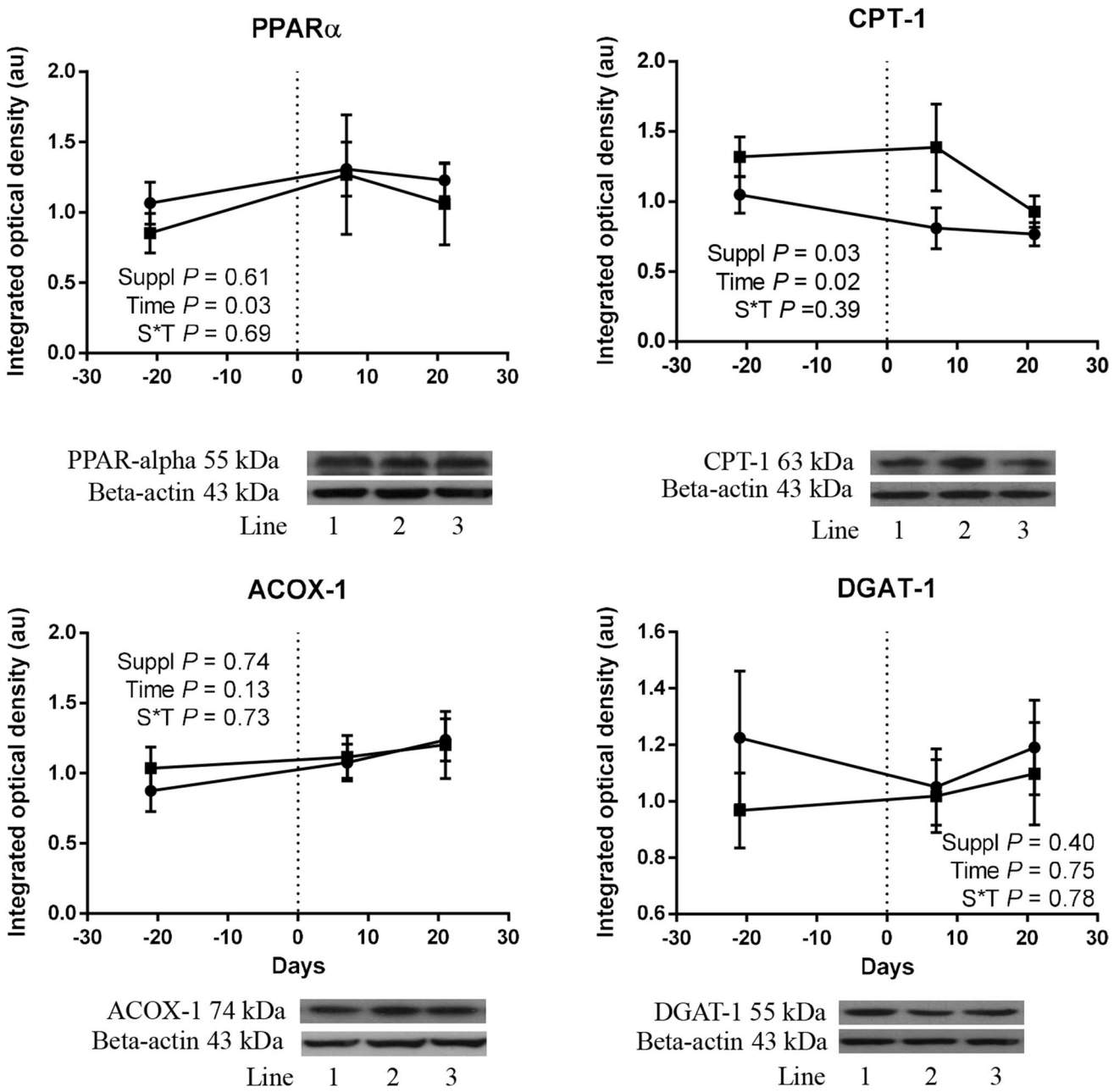

Fig. 2 Protein abundance of hepatic CPT-1 (**a**), ACOX-1 (**b**), DGAT-1 (**c**), and PPAR-α (**d**) between day 14 pre-calving and day 28 post-calving in animals grouped as supplemented cows (SG; $n = 11$; black squares) and control cows (CG; $n = 11$; black circles) at 21 days prepartum, and at 7 and 21 days postpartum. Top of each panel: Integrated optical density (au: arbitrary units) determined by Western blot analysis. Values are expressed as mean ± SEM. The statistical effects of supplementation (Suppl), time, and supplementation × time (S × T) are indicated. Bottom of each panel: Representative immunoblots of each determination; molecular weight is shown on the right. Lane 1, day 21 prepartum; lanes 2 and 3, days 7 and 21 postpartum. PPARα, peroxisome proliferator-activated receptor alpha; CPT-1, carnitine palmitoyltransferase 1; ACOX-1, acyl-CoA oxidase 1; DGAT-1, diacylglycerol O-acyltransferase 1

These results suggest that there would be no differences in the NEB between groups.

Another metabolite related to the NEB is glucose, and its concentrations have been shown to decrease postpartum as a result of the demand by the mammary gland for milk production [41]. In this study, the glucose concentration in the supplemented cows was higher despite their tendency to produce more milk. In dairy cows, the glucose supply depends predominantly on liver gluconeogenesis, and thus, alterations in their hepatic health, such as an increased fat content, may interfere with the capacity for hepatic glucose production during early lactation [42]. In this sense, we

may hypothesize that, in the absence of differences in the NEB parameters, such as in the concentrations of BHBA and NEFA, the lower concentration of systemic glucose could indicate an alteration in liver glucose synthesis. However, other analyses would be necessary to support this hypothesis.

Regarding hepatic health, CG cows had higher liver TAG content than SG cows. The TAG contents observed in the CG (2% TAG on a wet weight basis) usually do not affect milk production or DMI, but have been negatively correlated with health status and reproductive performance [2, 43]. Also, previous studies have indicated an association between the liver TAG content and the NEB [43, 44]. However, the results of our study showed no differences in the BCS or BHBA or NEFA concentrations between groups, thus suggesting that the difference observed in the liver TAG content was not associated with the NEB. These findings are consistent with a previous study of our group, in which we recorded a negative relation between greater liver TAG content and reproductive performance, without relation with the BCS or NEFA concentration [2]. Moreover, in this previous study, we observed a lower liver TAG content related to a greater oxidation capacity of fatty acids. Therefore, the liver of supplemented animals could be better adapted to the postpartum lipid mobilization.

Several studies have evaluated different biomarkers related to hepatic tissue damage and liver function [45, 46]. In this sense, in agreement with the results in the liver TAG content, albumin concentration was higher in the SG than in the CG. Regarding other biomarkers, particularly those related to hepatic tissue damage like AST and GGT, no differences were observed. Other studies have demonstrated no association between tissue damage parameters and plasma parameters related to liver function (such as bilirubin and albumin) in postpartum cows [28, 46, 47]. Albumin is considered an acute-phase protein of liver origin and some studies have found that, under pro-inflammatory situations, its serum concentration decrease [48]. The transition period has been described as a pro-inflammatory moment related to infectious diseases and fat mobilization [2, 49], and OS could also contribute to the development of a pro-inflammatory state [50]. In this sense, the supplementation with vitamins and minerals could have beneficial effect on the OS and inflammatory state, improving albumin synthesis and liver health.

Oxidative Stress Biomarkers

The minerals supplemented in this study are cofactors of antioxidant enzymes [51]. In this sense, we observed a significant increase of the GSH-Px activity in the SG, an important selenium-containing antioxidant enzyme, which could decrease the OS. The SOD activity and TAS were not be affected by supplementation. In this regard, Cortinhas et al. [24] described a beneficial effect of organic sources of Zn, Cu, and Se, reducing somatic cell count and the number of subclinical mastitis cases without modification in the antioxidant enzyme activities. Also, other study showed an increment in the SGH-Px activity with dietary antioxidant, but without changes in the SOD activity [52].

On the other hand, we measured the concentrations of 3-NT and MDA in liver. Only few studies in cows have demonstrated a relation between liver function parameters and OS [48, 53]. Moreover, we have recently showed a relation between reproductive performance and liver concentration of 3-NT and MDA in dairy cattle during the transition period [10]. In the present study, we observed a lower concentration of 3-NT in the liver of the SG without differences in liver MDA concentration. Bouwstra et al. [54] evaluated the concentration of liver MDA, another OS biomarker which is a degradation product of lipid peroxidation, and showed that it decreased postpartum in cows supplemented with vitamin E (3000 IU/day in the diet) and tended to increase in the control group. These previous results are in concordance with the beneficial effect of supplementation in the liver 3-NT concentration observed in our study, and which may be related to the beneficial effects on liver TAG content and albumin concentration previously described. In contrast, the concentration of 3-NT was higher in the plasma of the SG. In this sense, in a previous research of our group, we observed a high correlation between 3-NT concentration in urine and liver and a moderate correlation between 3-NT concentration in plasma and liver of dairy cattle [32]. It should be noted that the systemic concentrations of 3-NT could be influenced by the production of RONS by other organs, such as the mammary glands. Therefore, the differences obtained in plasma and liver could indicate that the oxidative status calculated based on blood values alone should be interpreted with caution and cannot be extrapolated to the whole animal. More studies will be necessary to further evaluate 3-NT as a possible biomarker of OS in dairy cattle. It is still intriguing whether the effect of vitamin-mineral supply on hepatic lipid metabolism is due to a modulation of inflammatory processes during the postpartum period or due to the antioxidant action associated with supplementation. Further studies will be necessary to better understand this issue.

Liver Fatty Acid Metabolism

On the other hand, several researchers have studied the effects of mineral and vitamin supplementation during the transition period on disorders, such as mastitis [55] and retained placenta [56], and on immunity [26]. However, we herein analyzed a possible association with relevant elements of hepatic fatty acid metabolism. To this end, in addition to measuring the liver TAG content, we determined the protein

abundance of relevant enzymes of hepatic fatty acid metabolism. Fatty acids are first converted into fatty acyl-CoA in the cytoplasm and then into acyl-CoA in the liver, probably through mitochondrial oxidation. Since some studies have recently shown that a rate-limiting step in the bovine mitochondrial lipid metabolism is the CPT-1 enzyme [57, 58], in this study, we evaluated CPT-1 protein abundance and found that it was higher in cows of the SG although there were no differences in the plasma NEFA concentrations between groups. By using human liver cells, Li et al. [59] showed that H_2O_2 suppresses *CPT-1* mRNA abundance. In addition, these authors described that cows with clinical ketosis had less liver *CPT-1* gene expression and other enzymes involved in the beta-oxidation of fatty acids. Similar results in *CPT-1* mRNA expression were observed in studies with cultured bovine hepatocytes with NEFA concentrations greater than 1.2 mmol/L [60]. Moreover, in a previous study, we described a lower CPT-1 protein abundance in cows with lower liver TAG content and better reproductive performance postpartum, but without relation to plasma NEFA concentration [2]. In this study, we found no evidence of ketosis in either group. However, the vitamin and mineral supplementation in cows could be beneficial for mitochondrial oxidation. In addition to the effect on *CPT-1* mRNA abundance, Li et al. (2012) described that H_2O_2 exposure also downregulates nuclear receptor *PPARα* expression and suppresses the expression of the target genes *ACOX-1* and *CPT-1*. In our study, no effect of supplementation was observed on the protein expression of PPARα. Previous studies by our group have also found no relationship between the protein abundance of PPARα and CPT-1 [2]. Also, some authors have evaluated PPARα and the important metabolic and inflammatory regulations in non-ruminants [61, 62], but these findings are not evident in ruminants [28]. Regarding peroxisomal oxidation, peroxisome oxidases could be affected by OS [1], but could also lead to the synthesis of H_2O_2, which suggests their participation in the metabolism of oxygen derivatives and their contribution to the oxidative phenomenon [16]. Even considering that the liver peroxisomal beta-oxidation of NEFA is greater (50%) in ruminants than in other species, in the present study, we found no differences in supplemented animals. This is in concordance with our previous findings showing that the ACOX-1 liver protein abundance is not related to reproductive performance [2]. No differences were found in the protein abundance of DGAT-1, a key enzyme in the liver synthesis of TAG. These results are in agreement with previous studies describing that the TAG content in the ruminant liver is related to the low capacity for secretion of very-low-density lipoproteins and not to the synthesis of TAG [63]. In this sense, we have previously described lower DGAT-1 protein abundance postpartum despite a higher liver TAG content [28].

Conclusion

In recent years, there has been a growing interest in the OS of dairy cows and in the supplementation with different antioxidants to improve their health and production. This study evaluated the effects of vitamin and mineral supplementation on the concentration of 3-NT in different biological samples, along with other plasma and liver parameters, to gain more insights into their possible relationship with fatty acid metabolism in the liver. In cows supplemented with vitamins and minerals, the liver had a lower concentration of 3-NT, higher expression of the mitochondrial beta-oxidation enzyme (CPT-1), and lower content of hepatic TAG, in concordance with the plasma liver function parameters herein studied. These results suggest that the supplementation had a beneficial effect on liver fatty acid oxidation during the peripartum. The results of 3-NT concentrations in the biological matrices analyzed were contradictory. These results could contribute for a better understanding of OS and its association with metabolic disorders in dairy cattle during the transition period.

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Author contributions Daiana Barcarolo and Emmanuel Angeli: Conceptualization, Methodology, Software, Data curation, Visualization, Investigation, Writing- Original draft preparation. Lucas E. Ribas and Lucas Etchevers: Conceptualization, Methodology, Software, Data curation, Writing- Original draft preparation. Valentina Matiller and Florencia Rey: Visualization, Investigation and Writing- Original draft preparation. Hugo H. Ortega and Gustavo J. Hein: Supervision- Writing- Reviewing and Editing.

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Declarations

Competing interests The authors declare no competing interests.

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