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Effect of parenteral trace element supplementation on oxidative stress and transcriptomic profile of peripheral blood in peripartum dairy cows

Calcaterra, F.[1,2]; Fernández, M.E.[1]; Olivera, L.H.[1]; Guzmán Loza, A.[3]; Giovambattista, G.[1]; Picco, S.J.[1*]

[1]IGEVET – Instituto de Genética Veterinaria "Ing. Fernando N. Dulout" (UNLP-CONICET-LA PLATA), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118 S/N, 1900, La Plata, Argentina. [2]Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, calles 60 y 118, 1900, La Plata, Buenos Aires, Argentina. [3]Cátedra de Patología Especial, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118 S/N, 1900, La Plata, Argentina.
✉ sjpicco@gmail.com

Abstract
The transition period is the most critical period in the lactation cycle of dairy cattle. During this period, cows can be exposed to high oxidative stress (OS), which can be managed through mineral supplementation with antioxidant micronutrients. The aim of this study was to evaluate the gene expression profiles of transition dairy cows supplemented with the antioxidant trace elements copper (Cu), zinc (Zn), manganese (Mn) and selenium (Se). The study was conducted in a commercial Holstein dairy farm located in General Belgrano, Buenos Aires, Argentina. Cows (n = 12) were randomly assigned to either a supplemented or a control group (n = 6 each). Blood samples were obtained on the day of calving and 4, 7 and 14 days after calving. Superoxide dismutase and glutathione peroxidase activity, antioxidant capacity (AC), and thiobarbituric acid reactive substances were determined. With these data, OS index was calculated. Additionally, RNA-sequencing analysis was performed on day +7, when OS index differences between groups were highest. The OS index and AC differed significantly between groups (day +7), despite only two differentially expressed genes codified for second messengers ($p \leq 0.05$). This would suggest that trace mineral supplementation in transition dairy cows does not induce changes in the gene expression profiles of pathways associated with OS and immune function, whose expression is already high in response to the high OS levels and the dietary changes during this period. Nevertheless, supplementation with the studied minerals improved the pro-oxidant/ anti-oxidant balance.

Key words: Dairy cows, oxidative stress, Transcriptome, Transition period, Mineral supplementation.

Análisis de la transcriptómica y del estado oxidativo en sangre de vacas lecheras suplementadas con minerales durante el período de transición

Resumen. El periodo de transición es la etapa más crítica en la lactancia de vacas lecheras, durante el cual los animales pueden estar expuestas a altos niveles de estrés oxidativo (EO), debido a sus funciones, la suplementación con minerales antioxidantes podría colaborar a su control. El objetivo de este trabajo consistió en evaluar los perfiles de expresión génica de vacas lecheras en el periparto suplementadas con los minerales antioxidantes cobre, zinc, manganeso y selenio. El estudio se llevó a cabo en un tambo comercial en la localidad de General Belgrano (Buenos Aires, Argentina). Los animales (n=12) fueron asignados al azar al grupo suplementado o al control (ambos con n=6). Las muestras de sangre se obtuvieron el día del parto (día 0) y en los días 4, 7 y 14 posteriores. Se midió la actividad de las enzimas superóxido dismutasa y glutatión peroxidasa, la capacidad antioxidante y las sustancias reactivas al ácido tiobarbitúrico. Con esta información se estimó el índice EO y también se realizó la transcriptómica mediante secuenciación de nueva generación (NGS) en el día +7, cuando la diferencia en el índice de EO entre ambos grupos era significativa. Del total de genes expresados, sólo hubo dos genes diferencialmente expresados, que

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codifican para segundos mensajeros. Esto sugiere que la suplementación con minerales antioxidantes de vacas lecheras en el periparto no induciría un aumento en la expresión de genes asociados cuya expresión ya se encuentra elevada debido a los altos niveles de EO y los cambios en la dieta asociados a este periodo. Sin embargo, la suplementación de estos minerales si mejoró el balance prooxidante / antioxidante.

Palabras clave: Ganado lechero, Estrés oxidativo, Transcriptoma, Período de transición, Suplementación mineral.

INTRODUCTION

The transition from gestation to lactation is the most critical phase in the lactation of dairy cattle. This phase, which includes three weeks before and three weeks after calving (Abuelo et al. 2015), is commonly named the transition period. In the last two decades, an increasing number of research have linked the peripartum of dairy cows with oxidative stress (OS) (Bernabucci et al. 2005), which is the result of an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity (AC) of the animal to neutralize them (Sies 2000). During the transition, OS may contribute to a number of health disorders, including mastitis, metritis, mammary edema and retained fetal membranes (Kankofer 2002, Sordillo and Aitken 2009), which can lead to decreased animal performance and health (Kankofer 2002, Durand et al. 2022). Therefore, it is not surprising that approximately 75% of diseases that affect dairy herds occur within the first month after calving, reaching maximum values within the first 10 days (Abuelo et al. 2015). While ROS are normally produced within cells, where they perform various biological functions, OS occurs when antioxidant systems are unable to counteract ROS increase (Abuelo et al. 2013). According to Celi (2011), more accurate information could be obtained by combining data referring to oxidants and antioxidants, rather than using them separately. As a separate evaluation of anti-oxidants and pro-oxidants may not evidence OS. However, when oxidative status is determined through OS index (OSi), which combines AC and TBARS values, differences may be significant. Considering that OS could be the result of a decrease in AC or an increase in ROS production, the relationship between these parameters needs to be taken into consideration (Abuelo et al. 2013). Also, Abuelo et al. (2013) suggest that the redox status of dairy cows can be assessed with the OSi, which is a combined measurement based on the ratio between antioxidants and ROS, also used to determine whether cows have experienced an oxidative challenge after calving.

One of the proposed strategies used for OS management is mineral supplementation with antioxidant micronutrients such as selenium (Se), an essential component of glutathione peroxidase (GSHpx) enzymes (Rotruck et al. 1973); copper (Cu) and zinc (Zn), both necessary for Cu-Zn superoxide dismutase (SOD) activity; and manganese (Mn) (Rotruck et al. 1973), which is important for Mn-SOD activity (Halliwell 1999). These minerals can boost the antioxidant response of the animals (Alhussien et al. 2021). They can also reduce the levels of stress (lower cortisol) and enhance health status by stimulating the immune system, resulting in lower somatic cell count, increased immunoglobulin content and phagocytic activity of neutrophils (Alhussien et al. 2021). Similarly, Warken et al. (2018) found an improved immune response, animals had lower somatic cell counts, reduced incidence of mastitis, an increase in T cells and neutrophil activity.

The complex metabolic phenotypes of the transition period can be investigated using high-throughput technologies (Loor 2010). In transition dairy cows, the adaptive changes in the gene expression profiles of different tissues and cells such as the liver (Loor et al. 2005, 2006, 2007), rumen (Steele et al. 2015), adipose tissue, mammary gland (Wang et al. 2015) and leukocytes (Minuti et al. 2020) have been identified using transcriptomics. Despite evidence suggesting that supplementation with antioxidant microminerals during transition provokes a differential antioxidant response (Abuelo et al. 2015) only a few studies have evaluated the effect of Cu, Zn, Mn and Se on the gene expression profiles of dairy cows during transition, although, these studies differ as Osorio et al. (2016) compared the effect of inorganic and organic supplementation of trace minerals on leukocytes and endometrium genes expression and Batistel et al. (2017) did the same with hoof cells.

Based on the hypothesis that transition dairy cows receiving a strategic supplementation of antioxidant microminerals have a lower OSi due to an improvement in the balance between the anti-oxidants (SOD, GSHpx, and AC) and pro-oxidants (TBARS), which results in differentially expressed genes (DEG) related with antioxidant pathways and stress response, the aim of the present work was to detect simultaneous changes in the OSi and the gene expression profiles of dairy cows parenterally supplemented with trace minerals (Cu, Zn, Mn and Se) during the transition period.

MATERIALS AND METHODS

Animals and experimental design. The study was conducted in a commercial dairy farm (Holstein herd size = 600; rolling average herd milk production = 9174 kg/cow/305 days) located in General Belgrano, Buenos Aires, Argentina (35°46′00″S 58°30′00″O). Lactating cows were fed a total mixed ration (TMR) *ad libitum*. Nutritional parameters and feed intake of dairy cows complied with the Nutrient Requirements of Dairy Cattle recommendations (Table 1 and 2; NRC 2001). Twelve Holstein cows meeting the following inclusion criteria were selected parity (2 to 5), not receiving parenteral supplementation with minerals 90 days before calving, and not receiving pharmacological treatment 60 days before calving. Cows were randomly assigned to two groups: supplemented intramuscularly (SG, n = 6) and control (CG, n = 6). SG animals received 5 mL of a mineral suspension containing Cu (10 mg mL^{-1} as edetate; Sigma-Aldrich®, Missouri, US), Zn (60

mg mL^{-1} as edetate; Sigma-Aldrich®), Se (5 mg mL^{-1} as sodium selenite; Sigma-Aldrich®) and Mn (10 mg mL^{-1} as edetate; Surfactan®) 20 days before calving (day -20) and on the day of calving (day 0). CG cows received 5 ml of sterile saline solution 20 days before calving and the day of calving. After calving, animals from CG and SG were grouped together and managed as a sole herd (Fresh Herd).

Table 1. Diet composition of dry and fresh Holstein cows (%). The used total mixed ration (TMR) complied with the Nutrient Requirements of Dairy Cattle recommendations (NRC 2001).

Component of the diet	Diet composition of dry cows (%)	Diet composition of fresh cows (%)
Corn Silage	73.54	64.12
Concentrate*	24.50	30.76
Anionic salts	1.96	5.12

*Based in a mix of Soybean meal and corn grain 17% of Crude Protein for dry cows, 20% for fresh cows, supplemented with minerals and vitamins.

Table 2. Predicted Nutrient analysis of dry and fresh Holstein cows. The used total mixed ration (TMR) complied with the Nutrient Requirements of Dairy Cattle recommendations (NRC 2001).

	Predicted nutrient analysis dry cows (%)	Predicted nutrient analysis fresh cows (%)
Dry Matter	65	53
Crude Protein	12	14.9
NDF	33	33
ADF	21	21
Calcium	0.44	0.79
Phosphorus	0.22	0.42

Blood samples were obtained by puncture of the jugular vein on days 0, +4, +7 and +14 to determine SOD and GSHpx activity, AC and thiobarbituric acid reactive substances (TBARS). They were collected in Vacutainer® EDTA tubes for hematocrit and GSHpx determination and in tubes without anticoagulant for colorimetric, SOD activity, AC and TBARS determinations. Blood subsamples at the moment of maximum OSi differences (day +7) were extracted, preserved in RNAlater (Thermo Fisher Scientific, USA) and stored at -80 °C until processing by RNA-sequencing. The study design is presented in Figure 1.

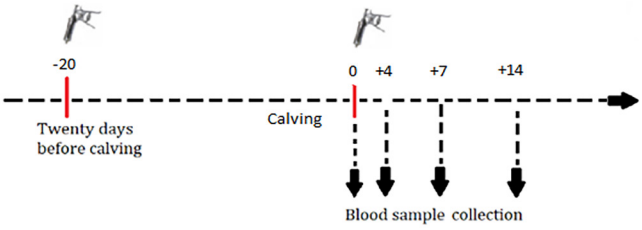

Figure 1. Experimental design. Red lines represent times of mineral supplementation. Black lines represent times of blood collection.

Biochemical determinations

GSHpx activity. Blood samples were used for erythrocyte hemolysis with high-performance liquid chromatography (HPLC)-grade water (1:4). They were frozen at -70 °C until complete sample collection (n = 12). After centrifugation, plasma was discarded and red blood cells were used to measure GSHpx activity by ultraviolet-visible (UV-vis) spectrophotometry with the Glutathione Peroxidase Assay Kit (Cayman Chemical Company, MI, USA) (340 nm wavelength), whose rate of decrease in A_{340} is directly proportional to GSHpx activity.

SOD activity, AC, and TBARS. After centrifugation, samples were aliquoted into two tubes, one for SOD activity and AC assessment and the other for TBARS determination. SOD activity was determined by UV-vis spectrophotometry using the Superoxide Dismutase Assay Kit® (Cayman Chemical). One SOD unit was the necessary amount of enzyme to produce a 50% reduction of the superoxide radical. Absorbance was measured at 440-460 nm. AC was determined by UV spectrophotometry with the Total Antioxidant Assay Kit (Cayman Chemical) at 750 nm. TBARS were determined with the TBARS Assay Kit (Cayman Chemical, Ann Arbor, Michigan) and measured at 530-540 nm.

OSi. The OSi was calculated according to Abuelo et al. (2013) with minor modifications. In the present work, OSi was estimated through the relationship between ROS and anti-oxidants. ROS were measured with the TBARS method which quantifies malondialdehyde (MDA), this assay was selected based on the fact that lipids are particularly sensitive to the attack of ROS (Celi 2011) and MDA is a common product of lipid peroxidation, which is assayed as an OS biomarker (Abuelo et al. 2015). And for the anti-oxidants we used AC which was mentioned previously. In Abuelo et al. (2013), OSi was defined as ROS / Serum antioxidant capacity (SAC). ROS were determined using the spectrophotometric d-ROM test (Diacron International, Italy), which determines hydroperoxides (breakdown products of lipids as well as of other organic substrate, generated by the oxidative attack of ROS) and SAC was estimated with the OXYAdsorbent Test (Diacron International, Italy) (Abuelo et al. 2013).

RNA-seq and differential gene expression (DGE) analysis. The blood samples preserved in RNAlater were delivered to the Novogene Sequencing Facility (Sacramento, CA, USA) for RNA extraction with assay and hemoglobin depletion. RNA quality was verified with an Agilent bioanalyzer (Agilent, CA, USA). After quality control procedures, the samples were sequenced in a NovaSeq 6000 sequencing platform (Illumina Inc, CA, USA) and the paired-end 150bp (PE150) strategy. Raw data were filtered to remove low-quality and adapter sequences. Then, the nf-core/RNAseq project pipeline (Ewels et al. 2020) was used to perform RNA-seq analysis using the ARS-UCD1.2 version as the reference bovine genome. In brief, this workflow includes the following analyses: quality control of reads with FastQC (Andrews

2010), STAR software for alignment to reference genome (Dobin et al. 2013), and featureCounts software for counting reads (Liao et al. 2014). Readings not having on average 10 total reads in SG and CG were filtered to delete non-expressed genes and transcripts with low expression levels. To identify differentially expressed genes (DEG), the results were analyzed through R software using the DESeq2 function (Love et al. 2014).

Ethics Approval. All animal procedures were performed by a veterinarian and reviewed and approved by the Institutional Committee on Care and Use of Experimental Animals from the School of Veterinary Sciences of the National University of La Plata (CICUAL; Buenos Aires, Argentina; Protocol 123-4-22T). Animals were handled according to the welfare guideline for Dairy cattle required by the CICUAL.

Statistical analysis. The biochemical determinations in SG and CG were compared using the Student´s t-test. Statistical significance was set at $p \leq 0.05$, and the power of the t-test was estimated using R studio package STATS. Results of this analysis are shown in supplementary Table S1. Identification of DEG was carried out using the Benjamini and Hochberg method implemented in the DESeq2 software in R (Love et al. 2014). Transcripts with an adjusted $p \leq 0.05$ were considered to have DGE, as identified by the Ensembl database.

RESULTS

Effects of mineral supplementation on phenotypic traits. The results of biochemical determinations showed that OSi and AC were significantly higher in CG than SG on days 0 and +7 ($p<0.05$). However, even though SOD and GSHpx activity levels were lower in CG on days 0, +7 and +14, differences between groups were not significant. Similarly, no differences were found in TBARS ($p>0.05$) (Table 3). Changes along the sampling period (0 to +14 days) of the significance variables are shown in Figure 2. These absence of significance values need to be validated using higher sampling size according to the t-test power analysis (Table S1).

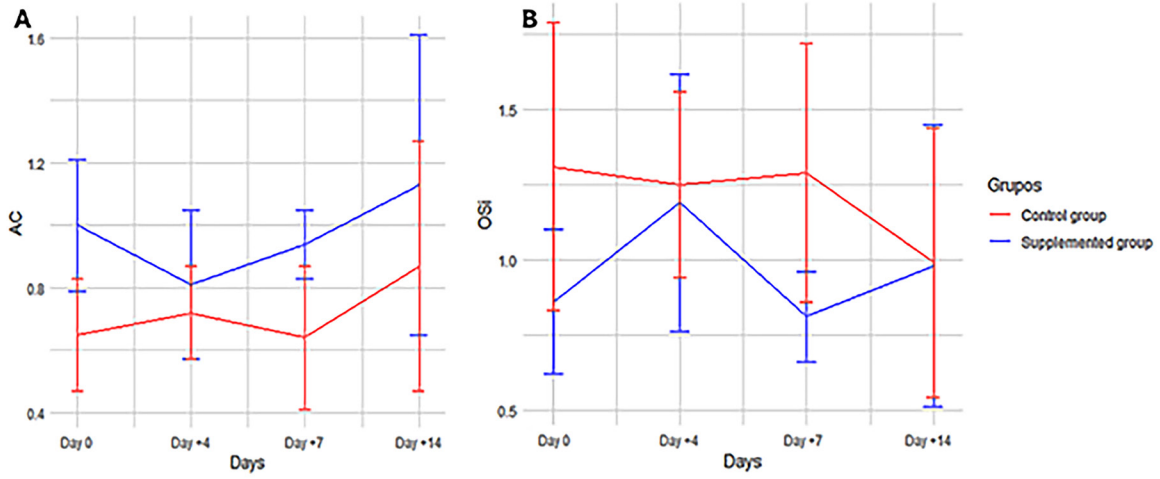

Figure 2. From day 0 (calving) to day +14 for supplemented and control group animals. **A.** Graph of antioxidant capacity (AC). B**.** Graph of oxidative stress index (OSi).

DGE analysis. The bioanalyzer measures evidenced that SG and CG samples exhibited an RNA integrity number (RIN) greater than 7.5 (average 8.40). RNAseq analyses of blood samples resulted in 116,969,995 million reads. Of these, 96.3% (% of global alignment) were successfully mapped to the ARS-UCD1.2. Low expression transcripts were filtered (< 10 reads in total), with 116,947,762 transcripts remaining for the following analysis. RNA-seq reads of the 12 blood samples from SG and CG detected a total of 27,429 genes in at least one of the samples. After filtering, 14,288 genes remained. Principal component analysis (PCA) based on the expression profiles showed that the two first components accounted for 82.1% of the total variance (PC1 = 70% and PC2 = 12.1%; Figure 3), without discriminating between SG and CG. The DGE analysis resulted in only two DEG (ENSBTAG00000034185 and ENSBTAG00000053198; adjusted $p \leq 0.05$) that encoded for guanosine triphosphate (GTP) protein activity.

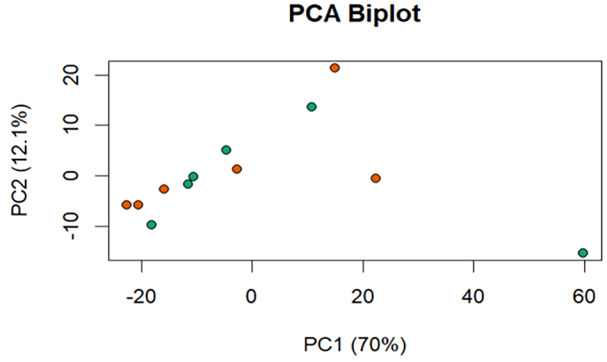

Figure 3. Principal component analysis showing the clustering of samples based on their gene expression profiles. Green and orange dots are represented by supplemented animals and control groups, respectively.

Table 3. Mean (± standard error of mean) of Glutathione peroxidase activity (GSHpx), Cu/Zn- Superoxide dismutase activity (SOD), Antioxidant Capacity (AC), t-BARs and Oxidative Stress index (Osi) in control and supplemented groups of dairy cows during the transition period. Animals from SG received 5 mL of a mineral suspension containing Cu (10 mg mL^{-1} as edetate; Sigma-Aldrich®, Missouri, US), Zn (60 mg mL^{-1} as edetate; Sigma-Aldrich®), Se (5 mg mL^{-1} as sodium selenite; Sigma-Aldrich®) and Mn (10 mg mL^{-1} as edetate; Surfactan®) 20 days before calving (day -20) and on the day of calving (day 0).

	GROUP	GSHpx U/ml PCV [a]	SOD U/ml	AC mM	TBARS nM MDA [b]	OSi AU [c]
Day 0	CG	70.29 (± 7.19)	0.59 (± 0.11)	0.65 (± 0.07)	0.8 (± 0.07)	1.31 (± 0.2)
	SG	76.51 (± 5.53)	0.610 (± 0.08)	0.99 (± 0.08)	0.81 (± 0.05)	0.86 (± 0.1)
	p value	*0.25*	*0.445*	***0.006***	*0.43*	***0.024***
Day +4	CG	81.63 (± 5.18)	0.60 (± 0.19)	0.721 (± 0.06)	0.87 (± 0.06)	1.25 (± 0.16)
	SG	74.36 (± 5.77)	0.51 (± 0.07)	0.812 (± 0.09)	0.89 (± 0.05)	1.19 (± 0.18)
	p value	*0.19*	*0.364*	*0.225*	*0.38*	*0.39*
Day +7	CG	80.38 (± 6.84)	0.41 (± 0.08)	0.64 (± 0.09)	0.77 (± 0.09)	1.29 (± 0.17)
	SG	85.44 (± 8.23)	0.52 (± 0.04)	0.94 (± 0.04)	0.75 (± 0.05)	0.81 (± 0.06)
	p value	*0.32*	*0.12*	***0.008***	*0.44*	***0.013***
Day +14	CG	82.693 (± 10.58)	0.497 (± 0.15)	0.87 (± 0.16)	0.71 (± 0.03)	1.09 (± 0.17)
	SG	86.26 (± 10.04)	0.595 (± 0.20)	1.13 (± 0.19)	0.95 (± 0.11)	0.98 (± 0.19)
	p value	*0.41*	*0.36*	*0.16*	***0.027***	*0.34*

Packed cell volume[a] (PCV), malondialdehyde[b] (MDA), arbitrary units[c] (AU)

DISCUSSION

In this study, supplementation with trace minerals improved the global antioxidant response of transition dairy cows, as shown by OSi. Even though differences in SOD and GSHpx activity levels and TBARS were not significant between groups, supplementation with Cu, Zn Se and Mn resulted in differences in AC and lower OSi on days 0 and +7 (Table 3), providing a more accurate and objective assessment of the animal oxidative status. A separate evaluation of anti-oxidants (SOD, GSHpx, and AC) and pro-oxidants (TBARS) showed that transition dairy cows did not, apparently, undergo OS. However, when oxidative status was determined through OSi, which combines AC and TBARS values, differences were significant. Considering that OS could be the result of a decrease in AC or an increase in ROS production, the relationship between these parameters needs to be taken into consideration (Abuelo et al. 2013). Thus, we observed that transition dairy cows that receive a strategic supplementation of antioxidant microminerals have a lower OSi than animals from CG, the next step to validate the hypothesis proposed above was analyzing the bulk gene expression profiles from both groups. This analysis did not show differences in gene expression (day +7), with the exception of two GTP protein activity genes ($p \leq 0.05$), whose expression was found to be higher in supplemented cows. These genes have a wide role as secondary messengers and are important regulators of gene expression and physiological changes such as proliferation, differentiation, migration, survival, apoptosis and depolarization (Takai et al. 2001).

The use of high-throughput technologies to characterize the different phenotypes of transition dairy cows has shown adaptive changes in the gene expression of different tissues. For instance, Minuti et al. (2020) found major changes after calving, which were mainly related to immunity, inflammation and endocrine aspects.

Omics technologies have also been used to study the effect of nutrient availability on the gene expression profiles of transition dairy cows. It has been reported that prepartum energy overfeeding of cows resulted in transcriptional changes, predisposing cows to fatty liver (Loor et al. 2006). Similarly, underfed cows also suffered changes in liver gene expression profiles (Loor et al. 2007). In cows receiving a daily oral bolus of a trace mineral complex (minerals linked to organic compounds) containing Cu, Zn, Mn and Co, DGE was related to inflammation and OS response in peripheral leukocytes and endometrial biopsies (Batistel et al. 2017) and hoof transcriptomics (Osorio et al. 2016) when compared with cows receiving inorganic supplementation.

As reported by different authors (Loor et al. 2005, Steele et al. 2015, Osorio et al. 2016, Batistel et al. 2017, Minuti et al. 2020), transition dairy cows naturally undergo changes in the expression of genes related to OS and immune function, concomitant with changes in their physiological state, metabolic requirements and the feed provided. Although these studies used different experimental models to compare leukocyte, hepatocyte and endometrial cell expression profiles, all works showed bulk transcriptome changes when comparing animals before and after parturition. Furthermore, there

are studies that evidenced that mineral supplementation increases antioxidant enzyme activity (Abuelo et al. 2015). However, the current results suggest that supplementation of transition dairy cows with trace minerals would not induce additional changes in the gene expression profiles of these pathways, this could be due to an already elevated expression in response to the high levels of OS and the metabolic and dietary changes associated with this period. Additional supplementation of minerals in dairy cows, which are fed according to NRC guidelines meaning basic mineral requirements are satisfied (As shown in Table 1 and 2), may not be necessary for an increased gene expression response. However, higher availability of these minerals in SG increased AC after calving as shown in Figure 2a, which could be partly explained by an improvement in antioxidant enzyme activity levels because they are necessary cofactors, although differences were not significant (Ma et al. 2017). Most of the antioxidant defenses are dependent on antioxidant enzymes using their specific substrates to reduce ROS. Enzymes like Cu/Zn or Mn-SOD and GSHpx constitute the first line of defense against free radicals of oxygen. Despite different strategies that have been used to prevent OS, including enzymes and small molecules acting as scavengers, the improvement of enzyme activity would be the most promising antioxidant therapeutic strategy (Forman and Zhang 2021). The ideas discussed above would be supported by the low number of DEG currently found in SG and CG. Additionally, although enzyme activity levels did not differ significantly, AC and OSi did, evidencing an improved antioxidant response, as shown in Figure 2a and b.

In conclusion, supplementation of transition dairy cows with antioxidant trace elements would be essential, considering that their gene expression profiles naturally change in response to the conditions of the transition period. However, changes in gene expression alone would not be enough to obtain an effective response, and the parenteral provision of micronutrients like Cu, Zn, Mn and Se would be fundamental to ensure an adequate antioxidant response, thus reducing the risk of OS and its possible consequences.

Data Availability. The transcriptomic data used in this study are available from GEO https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE231491.

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ORCID

Calcaterra F. https://orcid.org/0000-0001-8552-3203
Fernández M.E. https://orcid.org/0000-0003-4520-0607
Olivera L.H. https://orcid.org/0009-0006-3283-6197
Guzmán Loza A. https://orcid.org/0009-0008-5096-6053
Giovambattista G. https://orcid.org/0000-0001-8365-3303
Picco S.J. https://orcid.org/0000-0002-2904-2365

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Supplementary Table 1. n for a t test with 80% power and sample size required for a 80% power for each variable and moment.

	GROUP	**GSHpx U/ml PCV**	**SOD U/ml**	**AC mM**	**TBARS nM MDA**	**OSi AU**
Day 0	Sample size	96.32	2076.98	9.72	770.15	15.35
	power of t test 80%	*0.09*	*0.03*	*0.55*	*0.04*	*0.37*
Day +4	Sample size	53.91	266.27	78.49	566.08	566.08
	power of t test 80%	*0.13*	*0.05*	*0.1*	*0.04*	*0.04*
Day +7	Sample size	195.92	34.19	10.92	1135.12	11.94
	power of t test 80%	*0.06*	*0.18*	*0.5*	*0.03*	*0.47*
Day +14	Sample size	721.92	291.21	42.67	14.21	202.52
	power of t test 80 %	*0.04*	*0.05*	*0.15*	*0.14*	*0.06*