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Effect of injectable trace mineral supplementation on peripheral polymorphonuclear leukocyte function, antioxidant enzymes, health, and performance in dairy cows in semi-arid conditions

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ABSTRACT

The objective of this study was to evaluate the effect of subcutaneous injections of 15 mg/mL Cu, 5 mg/mL Se, 60 mg/mL Zn, and 10 mg/mL Mn on health, performance, polymorphonuclear leukocyte (PMNL) function, circulating glutathione peroxidase (GPx) and superoxide dismutase (SOD) concentrations, and inflammation of dairy cows undergoing the transition period in high temperature-humidity index. A total of 923 multiparous cows from 2 commercial dairy farms were randomly allocated into 1 of 2 treatment groups as follows: control and injectable trace mineral supplementation (ITMS). Cows in the ITMS group received 7 mL of subcutaneous injections at dry-off (208 ± 3 d of gestation), 260 ± 3 d of gestation, and at 35 ± 3 d in milk (DIM). Data regarding health traits, reproductive performance, milk yield, and survivability were extracted from farm database software, and animals were followed-up until 300 DIM. For a subset of 142 cows from one herd, blood samples were collected at enrollment, and at 3 ± 1, 7 ± 1, 10 ± 1, and 35 ± 3 DIM to evaluate hematology, PMNL function, GPx and SOD concentrations, and circulating haptoglobin. Logistic regression was used to assess health and pregnancy per artificial insemination at first service. Cox proportional hazards models were used to evaluate hazard of pregnancy and culling. Mixed linear regression models accounting for repeated measures were used to assess all continuous variables collected over time. Parity, twinning, and previous gestation length were considered as potential confounders. Farm was included as a random effect. The ITMS cows tended to have lower incidence of metritis and stillbirth compared with control group. However, ITMS treatment did not influence the incidence of other diseases (e.g., mastitis, retained placenta), milk yield, reproductive performance, culling, and leukocyte count. Neutrophil-to-lymphocyte ratio, PMNL phagocytosis, and oxidative burst as well as intensity of the oxidative burst were greater for ITMS-treated cows in comparison to control cows. The ITMS cows had decreased expression of the adhesion molecule L-selectin on PMNL surface. The serum concentration of GPx and SOD were not affected by ITMS treatment. In conclusion, ITMS tended to reduce the incidence of metritis and stillbirth parturition, improved PMNL function, and improved the inflammatory status of dairy cows undergoing the transition period in high temperature-humidity index conditions. However, these findings did not translate into improved milk yield, reproductive performance, and survivability.
Key words: trace mineral, dairy cow, immune function, health

INTRODUCTION

During the transition period, from 3 wk before to 3 wk after parturition, dairy cows face an impairment or dysregulation of their immune function, which has been recognized as a central element to the development of diseases, such as metritis and mastitis (LeBlanc, 2020). Concurrently, during the periparturient period, dairy cows experience a substantial increase in oxygen consumption that results in elevated production of reactive oxygen species (**ROS**; Sordillo, 2016). Therefore, an imbalance between the production of ROS and the availability of antioxidant defenses may expose these animals to oxidative stress (Sordillo and Aitken, 2009). Additionally, heat stress has been reported to aggravate this scenario, whereas cows can experience greater degrees of feed intake depression (Adin et al., 2009), increased degree of oxidative stress (Bernabucci et al., 2002; Safa et al., 2019), and immune dysregulation (Lacetera et al., 2005; Lecchi et al., 2016; Safa et al., 2019). High proportion of puerperal disorders have been observed in dairy cows undergoing the transition

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period in heat-stress conditions (Gernand et al., 2019). Heat stress in dairy cows is particularly important in dairy herds located in semi-arid regions. For instance, in the southwest region of the High Plains in the United States or Northern Mexico, where the dairy industry is an important part of the local economy, the average temperature-humidity index (**THI**) during the summer is above 68, a number commonly used as the threshold for stress in dairy cows (Zimbelman et al., 2009).

Thermal environmental conditions may affect the immune system by complex mechanisms including body temperature change, behavioral and hormonal adaptation, circulatory adjustment, and oxidative stress (Lacetera, 2012). Shi et al. (2020) found significant changes of immunoglobulin and cytokine concentration and immune-related gene expression in heat-stressed lambs. Additionally, the authors detected reduced antioxidant enzyme activity coupled with oxidative stress status, which might be through ROS overproduction caused by hyperthermal stress. Providing shade and soaking lines are strategies commonly adopted to improve the performance and immune status of postpartum dairy cows undergoing heat stress (do Amaral et al., 2011). Also, supplementation of Zn, Mn, Cu, and Se, which are components of antioxidant enzymes such as glutathione peroxidase (**GPx**) and superoxide dismutase (**SOD**), have been studied as alternative strategies to alleviate detrimental effects of heat stress on immunity, antioxidant status, and performance of chicken (Abd El-Hack et al., 2017; Rajkumar et al., 2018) and sheep (Alhidary et al., 2015). Hence, it is plausible that trace mineral supplementation can also help to mitigate the adverse effects caused by the burden of heat stress in other livestock such as dairy cows. Because of dietary deficiency of Zn, Cu, Mn, and Se in dairy cows, either through antagonisms or imbalanced diets, the supplementation of these trace minerals has been intensively studied in dairy cattle (Overton and Yasui, 2014).

The use of injectable trace mineral supplementation (**ITMS**) with Zn, Mn, Cu, and Se improved health (Machado et al., 2013) and serum SOD concentration of dairy cows (Machado et al., 2014) when supplemented at dry-off, 30 d before calving, and during the second month of lactation. However, Bicalho et al. (2014) did not detect differences in GPx and SOD activities when administrating 2 injections of trace minerals at 230 and 260 d of gestation. Additionally, postpartum polymorphonuclear leukocyte (**PMNL**) function, milk yield, and reproductive performance were not influenced by ITMS. Those studies were performed in dairy herds located in New York during cold-weather conditions. Because the concentration of ROS-scavenging trace mineral–dependent enzymes may increase under heat-stress conditions (Rhoads et al., 2013), ITMS in cows under heat stress could lead to different outcomes.

Therefore, the objective of this study was to evaluate the effect of an ITMS containing Cu, Se, Zn, and Mn at dry-off (208 ± 3 d of gestation), 260 ± 3 d of gestation, and 35 ± 3 d postpartum on incidence of postpartum diseases, milk yield, reproductive performance, culling, hematological parameters, PMNL activity, GPx and SOD concentrations, and circulating haptoglobin (**Hp**) in dairy cows undergoing the transition period in high THI.

MATERIALS AND METHODS

Farms, Management, and Environmental Data

All experimental procedures were approved by the Texas Tech University Institutional Animal Care and Use Committee (#19045–05). This study was conducted on 2 commercial Holstein dairy farms (farm A and farm B) located in west Texas. Farms were selected based on their geographical location (near Lubbock, TX), and willingness to participate in the study. Cows were enrolled from April 30 until July 23, 2019; the follow-up period continued until November 13, 2019. The enrollment period was selected to maximize the number of days with THI >68 during the transition period. The environmental information was assessed from the Lubbock Airport's meteorological station because of its proximity to both dairies (67 and 48 km from farms A and B, respectively). Daily temperature and relative humidity data were downloaded from Weather Underground (https://www.wunderground.com/history/daily/us/tx/lubbock) from April 30 through November 13, 2019. The THI throughout the study period was calculated from the equation developed by the National Research Council (NRC, 1971) below as indicated by Dikmen and Hansen (2009):

$$\text{THI} = (1.8 \times \text{T} + 32) - [(0.55 - 0.0055 \times \text{RH}) \times (1.8 \times \text{T°C} - 26)],$$

where T = ambient dry bulb temperature in °C, and RH = relative humidity in %. Meteorological data are presented in Figure 1.

Farm A milked 2,700 Holstein cows twice a day in a double 30-stall parallel milking parlor. The cows were housed in dry-lot pens, consisting of nonvegetated open lots (i.e., corrals) with shaded areas. Farm B milked 3,700 Holstein cows 3 times daily in a 70-stall rotary milking parlor. During the dry period, the animals were housed in dry-lot pens, consisting of nonvegetated open lots. After calving, the cows were housed in freestall

Table 1. Chemical composition of pre-fresh and fresh cow diets (% of DM, otherwise stated)

	Farm A		Farm B	
Item	Pre-fresh[1]	Fresh[2]	Pre-fresh	Fresh
DM, %	59.2	47.8	47.2	47.8
CP	14.8	18.7	13.1	17.6
Soluble protein, % CP	43.8	34.1	32.6	34.6
ADF	23.5	20.6	28.5	17.4
NDF	35.2	31.4	39.2	27.7
NFC	37.8	36.4	34.1	41.9
Starch	24.9	23.9	24.5	27.0
Calcium	1.33	0.93	0.73	0.99
Phosphorus	0.34	0.44	0.3	0.37
Magnesium	0.42	0.37	0.28	0.35
Potassium	1.7	1.53	1.09	1.62
Sodium	0.17	0.34	0.13	0.74
Sulfur	0.25	0.3	0.25	0.22
Chloride	1.78	0.69	0.5	0.73
Iron, mg/kg	437	366	978	469
Zinc, mg/kg	103	57	49	104
Copper, mg/kg	27	15	8	18
Manganese, mg/kg	92	51	49	57
Selenium, mg/kg	0.62	0.43	0.39	0.62

[1]Pre-fresh diets were fed from 3 wk prepartum through parturition.
[2]Fresh diets were fed from parturition through wk 35 postpartum.

barns with concrete stalls and bedded with manure solids without fans and soaking lines. At both farms, the animals were fed a TMR ad libitum with free access to water. According to herd characteristics, the diets were formulated to meet or exceed the NRC (2001). Composite TMR samples from pre-fresh and lactation diets were submitted to a commercial laboratory (Cumberland Valley Analytical Services Inc., Waynesboro, PA). Near-infrared spectroscopy was used to measure DM, CP, ADF, and NDF. Macro and trace minerals, except Se, were analyzed by inductively coupled plasma MS (AOAC International, 2000; method 985.01). Selenium was assessed by fluorometric method (AOAC International, 2005; method 996.16). Nutrient contents of the diets are described in Table 1.

Sample Size Calculation

Sample size calculation was undertaken in MedCalc version 18.11.6 (MedCalc Software). Based on previously reported data (Machado et al., 2013), we assumed that ITMS would decrease the incidence of clinical

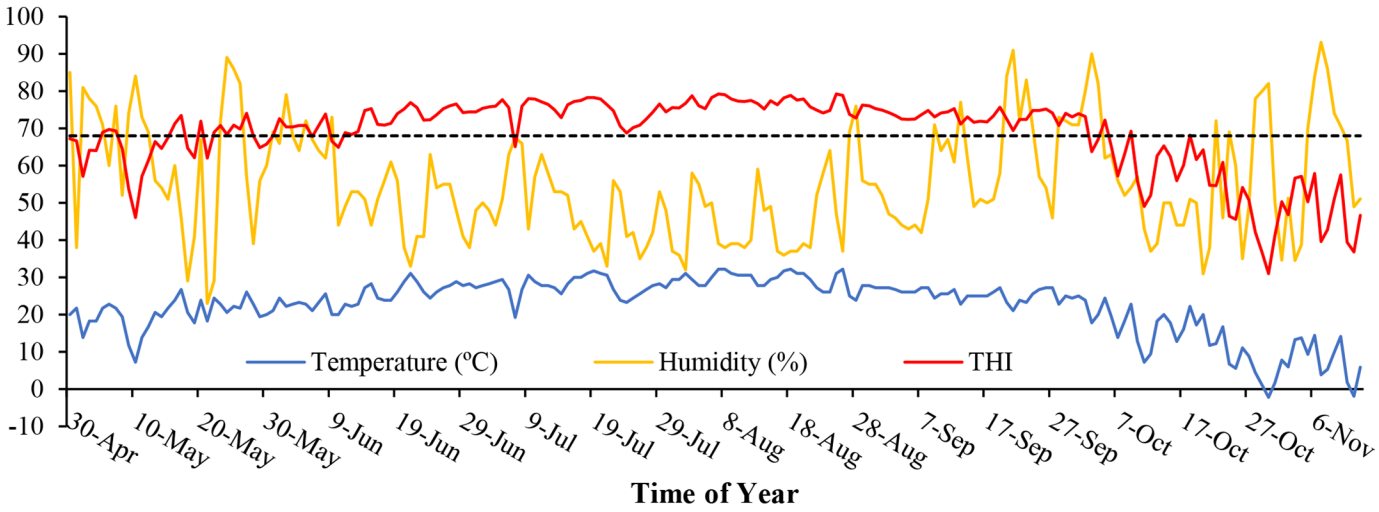

Figure 1. Daily mean environmental ambient temperature (°C), relative humidity (%), and temperature-humidity index (THI) throughout the study from April 30 to November 13, 2019, in west Texas. Dotted lines indicate THI threshold for heat-stress conditions for dairy cows.

mastitis from 26 to 18%. Considering a significance level of $\alpha = 0.05$ and a power of 80%, at least 420 cows would have to be enrolled in each treatment group. Accounting for 10% attrition, 956 animals were enrolled in the study (n = 462 and n = 494 for control and ITMS group, respectively). With this sample size, we were able to detect a milk yield difference between the groups of 1.1 kg/d with standard deviation of 6 kg/d, considering $\alpha = 0.05$ and a power of 80%. For blood analysis, based on significant differences detected by Nightingale et al. (2015) and Silva et al. (2020) with regard to the proportion of PMNL that could perform oxidative burst during the early postpartum period, we expected that the proportion of PMNL that could perform oxidative burst would be 8 percentage points greater in the ITMS group compared with cows in the control group. Considering a standard deviation of 15 and assuming $\alpha = 0.05$ and a power of 80%, at least 60 cows in each group would be needed.

Inclusion Criteria, Treatment Allocation, Case Definitions, and Data Collection

Dry cows were included in the study based on expected calving date (between July and September). A randomized clinical trial blocked by farm included a total of 923 multiparous cows (from 956 animals enrolled in the study, 33 of them were excluded due to loss to follow-up). At dry-off, cows were randomly allocated into 1 of 2 treatment groups as follows: ITMS or control. Randomization was completed in Excel 2019 (Microsoft Corp.) and imported into the farm's management software program as treatments A and B. Farm personnel were unaware of the coding and were blinded to treatment allocation. Cows that were randomly assigned to the ITMS group received 7 mL of subcutaneous injections of 15 mg/mL Cu, 5 mg/mL Se, 60 mg/mL Zn, and 10 mg/mL Mn (Multimin 90, Multimin North America) at dry-off (208 ± 3 d of gestation), 260 ± 3 d of gestation, and at 35 ± 3 DIM. Pregnant heifers were not enrolled in the study because ITMS was not beneficial to cows entering the lactating herd (Machado et a., 2013). The ITMS administration was performed by the research team.

Postpartum diseases were diagnosed and treated by trained farm personnel who were blinded to treatments. Metritis was defined as the presence of fetid, watery, red-brown uterine discharge (Sheldon et al., 2006). Clinical mastitis was defined by the diagnosis of abnormal changes in the udder or milk (Lago et al., 2011). Retained placenta (**RP**) was defined as the failure to release fetal membranes within 24 h of calving (Kelton et al., 1998). Stillbirth parturition was defined as the sum of stillbirth event + intrapartum fetal death + early neonatal death. Thus, the term stillbirth encompassed the death of a calf occurring just before, during, or within 48 h of parturition (Philipsson et al., 1979). Data regarding health traits, reproduction outcomes, milk yield, and survivability were extracted from Bovisynch (Dairy LLC) and DairyComp 305 (Valley Agricultural Software) databases on farms A and B, respectively, up to 300 DIM.

Blood Collection and Analysis

For a subset of 142 cows from farm A, blood samples were collected for differential blood cell count purposes, PMNL function, GPx and SOD concentrations, and circulating Hp. Blood samples were collected at enrollment and at 3 ± 1, 7 ± 1, 10 ± 1, and 35 ± 3 DIM by puncture of the coccygeal vessels using a 10-mL Vacutainer tube without anticoagulant, a 10-mL Vacutainer tube with lithium heparin, a 3-mL Vacutainer tube with potassium EDTA, and a 20-gauge × 2.54-cm Vacutainer needle (Becton, Dickinson and Co.). After collection, the heparinized blood was stored in an ice chest with no ice at ambient temperature to preserve their phagocytic and oxidative burst capacity (Sellers et al., 2013). The EDTA and coagulated blood samples were immediately placed on ice. Samples were analyzed or processed within 3 h after collection. The blood samples without anticoagulants were centrifuged at 2,000 × *g* for 15 min at 4°C for serum separation, and frozen at −80°C. The heparinized and EDTA blood samples were processed in the same day of collection for measures of differential blood cell count and ex vivo PMNL responses.

Leukocyte count and differentials were performed using a hematology analyzer (Idexx Procyte DX), and the variables of interest were total leukocytes, monocytes, neutrophils, lymphocytes, and neutrophil-to-lymphocyte ratio. Flow cytometry was used to determine the phagocytosis and oxidative burst capacity of PMNL and the quantification of their adhesion molecule L-selectin (CD62 L). Both procedures were performed as previously described by Hulbert et al., (2011) with minor protocol modifications. To measure the phagocytic and oxidative burst capacity, 100 μL of heparinized whole blood were incubated for 15 min in an ice bath. After this initial incubation, 20 μL of a 100 μ*M* solution of dihydrorhodamine (Invitrogen) and 20 μL of a 10^9 cfu/mL propidium iodide–labeled *E. coli* suspension were added to the blood and then incubated in a 38.5°C water bath for 10 min (negative controls were incubated in an ice bath for 10 min). Then, samples were immediately placed on an ice bath for 5 min, and erythrocytes were hypotonically lysed and washed with PBS. Dual-color flow cytometry was performed using an Attune flow

cytometer (Life Technologies/Thermo Fisher Scientific Inc.). The PMNL population was gated using forward and side scatter plots. The mean fluorescence intensity and proportion of PMNL that performed phagocytosis and oxidative burst were acquired using the optical filters BL3 (excited by a 488-nm laser on a 695/40 filter) and BL1 (excited by a 488-nm laser on a 530/30 filter), respectively. Negative controls were used to determine negative and positive signals on the BL1 by BL3 scatterplot used to assess PMNL that performed phagocytosis and oxidative burst. To determine the expression of the adhesion molecule L-selectin, 50 μL of EDTA blood samples were mixed with 50 μL of PBS containing 1 μg of a monoclonal antibody mouse IgG1-isotype (catalog number: BOV2046, clone: BAQ92A; Veterinary Microbiology and Pathology Monoclonal Antibody Center). After a 1-h incubation in an ice bath, erythrocytes were hypotonically lysed. After centrifugation (1,200 × *g* for 5 min at 4°C), the leukocyte pellet was resuspended in 50 μL of fluorescein-labeled secondary antibody at a 1:400 dilution [F(ab′)2 anti-mouse IgG:FITC; AbD Serotec] and incubated for 1 h in an ice bath. After a PBS wash, samples were analyzed using single-color flow cytometry. The PMNL population was gated as previously described, and the mean fluorescence intensity for L-selectin was gathered using BL-1. An unconjugated IgG1 isotype control was used to determine minimal nonspecific binding of the primary antibody used to determine CD62L (L-selectin) expression in bovine whole blood. The use of F(ab′)2 secondary conjugates and the use of secondary only controls confirmed minimal nonspecific binding. Data were analyzed using Attune N×T cytometric software, version 3.1.2 (Life Technologies/Thermo Fisher Scientific Inc.).

Commercial kits (Cayman Chemical) were used to assess the following serum antioxidant enzymes: GPx (#703102) and SOD (#706002). Serum Hp concentration was determined using a colorimetric assay via quantification of the Hp-hemoglobin complex by the estimation of differences in peroxidase activity (Makimura and Suzuki, 1982). Assays were performed in 16 × 100 borosilicate tubes. Briefly, 5 μL of serum sample or deionized water (blank) was added to 7.5 mL of a solution containing 0.6 g/L *o*-dianisidine, 13.8 g/L sodium phosphate monobasic, and 0.5 g/L EDTA (pH = 4.1). Immediately, 25 μL of 0.3 g/L bovine hemoglobin solution was added to each assay, followed by a water bath incubation at 37°C for 45 min. After incubation, 100 μL of freshly prepared 156 m*M* hydrogen peroxidase solution was added to each assay. Samples were incubated at room temperature for 60 min. Then, 200 μL of each assay was transferred to a 96-well polystyrene flat-bottom microplate. Optical density at 450 nm was measured on the Epoch2 Microplate Spectrophotometer (BioTek Instruments Inc.). Finally, the final optical density of each assay was subtracted by the blank assay values. Optical density data were converted to concentration units (μg/mL) using standard curves generated by serial dilutions of a sample of known concentration determined by a commercially available ELISA kit following the manufacturer's instructions (Life Technologies) as previously described (Cooke and Arthington, 2013). The intra and interassay coefficient of variation for serum Hp were 1.8% and 5.9%, respectively.

Statistical Analysis

All statistical analyses were performed in SAS version 9.4 (SAS Institute Inc.). Descriptive statistics analysis was undertaken using the FREQ procedure. The odds of metritis, clinical mastitis, RP, stillbirth, and pregnancy per AI (**P/AI**) at first service was assessed by fitting the data using the GLIMMIX procedure. The effect of treatment on reproduction and survival was analyzed by Cox's proportional hazard using the PHREG procedure. The proportional hazards assumption was tested visually through plots of the scaled Schoenfeld residuals using the model as a function of time. For analysis of reproduction, cows were right-censored if not diagnosed pregnant before culling, death, or the end of the data-collection period. For all models described above, the variables offered included treatment, parity (2, >2), twins, and previous days of gestation. The independent variables and their respective interactions were kept when $P < 0.10$. The models included the fixed effect of treatment, which was forced into all models, and farms, which were included as random effect. Adjustments for multiple comparison were not included for the logistic regression and Cox's proportional hazard models because the only variable of interest was treatment, and the other independent variables retained in the model were for controlling for confounding purposes. To illustrate the median calving-to-conception interval, and the median time to culling or death for each treatment group, Kaplan-Meier survival analysis was performed using MedCalc; the Logrank test was used to compute *P*-values.

The effect of ITMS on monthly milk yield during the first 180 DIM was evaluated through a repeated measures model fitted by multiple mixed linear models using the MIXED procedure. The model included the fixed effects of treatment, month of lactation, parity (2, >2), twins, and previous days carried calf. The independent variables and their respective interactions were kept when $P < 0.10$. The farm variable was included as a random effect. The normality of the residuals was analyzed with normal probability and box plots visualization. To account appropriately for within-cow

Table 2. Descriptive statistics of treatment groups[1]

Item	Control	ITMS
Farm A		
Total number of enrolled animals (%)	253 (50)	252 (50)
Enrolled animals on parity 2 (%)	81 (48)	87 (52)
Enrolled animals on parity >2 (%)	172 (51)	165 (49)
Average days of gestation at enrollment (±SE)	207.8 (4.8)	208.2 (4.8)
Subset of animals for blood evaluation		
Total number of enrolled animals (%)	74 (52)	68 (48)
Enrolled animals on parity 2 (%)	26 (55)	21 (45)
Enrolled animals on parity >2 (%)	48 (51)	47 (49)
Average days of gestation at enrollment (±SE)	208.3 (1.6)	208.1 (2.0)
Farm B		
Total number of enrolled animals (%)	196 (47)	222 (53)
Enrolled animals on parity 2 (%)	92 (45)	114 (55)
Enrolled animals on parity >2 (%)	104 (49)	108 (51)
Average days of gestation at enrollment (±SE)	209.6 (5.2)	209.2 (5.2)
Total enrolled animals on farms A and B (%)	449 (49)	474 (51)
Enrolled animals on parity 2 (%)	173 (46)	201 (54)
Enrolled animals on parity >2 (%)	276 (50)	273 (50)

[1]Control cows received no trace mineral injection; ITMS cows received 7-mL subcutaneous injections of 15 mg/mL Cu, 5 mg/mL Se, 60 mg/mL Zn, and 10 mg/mL Mn (Multimin 90, Multimin North America) at dry-off (208 ± 3 d of gestation), 260 ± 3 d of gestation, and at 35 ± 3 DIM.

correlation, the error term was modeled by a compound symmetry covariance structure due to its smallest Bayesian information criterion value.

To evaluate the effect of ITMS on leukocyte counts, PMNL phagocytosis and oxidative burst activity, SOD, GPx, and Hp throughout the 5 blood collection days (enrollment, 3, 7, 10, and 35 d relative to calving), repeat measures models were fitted by multiple mixed linear models using the MIXED procedure. The experimental unit was the cow. The normality of the residuals was analyzed with normal probability and box plots visualization. To account appropriately for within-cow residues correlation, a spatial (power) covariance structure (SP[POW]) was applied for all models. This variance-covariance structure is indicated for unequally spaced data collection and assumes correlations decline as a function of time. For multivariate models above, independent variables and their respective interactions were kept when $P < 0.10$. The effect of treatment, time, and their interaction were forced into all statistical models even in the absence of statistical significance. The SLICE option using a Bonferroni multiple comparison adjustment was used to explore interactions between treatment and time whenever the F-test was significant. Days of gestation at enrollment, parity (2, >2), and the value of the dependent variable assessed at enrollment were offered to all models as a covariate to control confounding effects. For all the analyses, differences detected at $P \leq 0.05$ were considered significant, and differences at $0.05 < P < 0.10$ were considered a tendency toward statistical significance.

RESULTS

Descriptive Statistics

A total of 956 animals were enrolled in the study. From these, 33 cows were excluded due to loss to follow-up, with 24 cows being from farm A (control group = 11 and ITMS = 13) and 9 cows being from farm B (control group = 2 and ITMS group = 7). A total of 923 cows from the initial 956 remained in the study. Descriptive statistics regarding the number of animals enrolled in parity 2 and >2 and average gestation length (days) at enrollment are presented in Table 2.

Effect of ITMS on Health, Reproductive Performance, Milk Yield, and Culling

The effect of treatment on health and P/AI at first service is presented in Table 3. Briefly, ITMS-treated cows tended to have lower incidence of metritis compared with control group [odds ratio (**OR**) = 0.68, 95% CI = 0.4–1.0, $P = 0.051$]. Additionally, administration of ITMS tended to reduce the incidence of stillbirth compared with cows in control group (OR = 0.41, 95% CI = 0.17–1.01, $P = 0.052$). However, supplementing cows with injectable trace minerals did not improve the odds of mastitis ($P = 0.379$) and RP ($P = 0.657$) compared with unsupplemented cows.

Injectable trace mineral supplementation did not affect reproductive performance. The treatment did not improve P/AI at first service (OR = 1.03, 95% CI =

Table 3. Effect of treatment on incidence (%) of peripartum diseases and first-service conception

Item	Treatment[1]		Odds ratio (95% CI)	*P*-value
	Control (%)	ITMS (%)		
Metritis	12.6	8.6	0.63 (0.40–1.0)	0.051
Mastitis	30.4	32.0	1.14 (0.85–1.53)	0.379
Retained placenta	8.7	7.7	0.89 (0.54–1.47)	0.657
Stillbirth	3.7	1.5	0.41 (0.17–1.01)	0.052
First-service conception	35.4	36.0	1.03 (0.76–1.39)	0.874

[1]Control cows received no trace mineral injection; ITMS cows received 7-mL subcutaneous injections of 15 mg/mL Cu, 5 mg/mL Se, 60 mg/mL Zn, and 10 mg/mL Mn (Multimin 90, Multimin North America) at dry-off (208 ± 3 d of gestation), 260 ± 3 d of gestation, and at 35 ± 3 DIM.

0.76–1.39, $P = 0.874$) or hazard of pregnancy up to 150 DIM compared with control cows [hazard ratio (**HR**) = 0.99, 95% CI = 0.86–1.14, $P = 0.901$]. Additionally, no improvements in milk yield and culling were observed. The average milk yield during the first 6 mo of lactation for cows enrolled in control and ITMS group was 43.9 kg/d and 43.4 kg/d, respectively ($P = 0.177$), and there was no interaction between treatment and month of lactation ($P = 0.925$). Furthermore, the hazard of culling up to 300 DIM was similar between the groups (HR = 1.25, 95% CI = 0.96 – 1.65, $P = 0.103$). The survival curves for time-to-pregnancy and days to culling or death by treatment group is presented in Figure 2.

Effect of ITMS on Hematology, PMNL Function, Antioxidant Enzymes, and Hp

The effect of ITMS on total leukocyte, monocyte, neutrophil, and lymphocyte counts, and neutrophil-to-lymphocytes ratio are presented in Table 4. The ITMS treatment did not influence the total leukocytes, neutrophil, and lymphocyte counts during the study. However, neutrophil-to-lymphocyte ratio was greater for ITMS-treated cows in comparison to control cows throughout the study ($P = 0.039$). Additionally, ITMS did not influence the GPx, SOD, and Hp concentrations after calving ($P = 0.966$, $P = 0.320$, $P = 0.210$ for GPx, SOD, and Hp, respectively; Table 4).

The ITMS treatment improved the PMNL function during the postpartum period due to increased proportion of PMNL that performed phagocytosis ($P = 0.005$) and oxidative burst ($P = 0.001$) and tended to increase the intensity of oxidative burst ($P = 0.051$; Figure 3A, B, D). Additionally, the expression of L-selectin on PMNL surface was decreased in ITMS cows ($P = 0.035$; Figure 3E).

DISCUSSION

In this study, ITMS-treated cows undergoing the transition period in THI >68 had improved PMNL function early postpartum. Accordingly, a reduction in the incidence of diseases was expected for supplemented cows in comparison to control cows. Nevertheless, only a numerical reduction in metritis and stillbirth incidence was detected, and no effect was observed of ITMS on the incidence of RP and mastitis. Machado et al. (2013) reported that ITMS improved udder health by decreasing the odds of clinical mastitis and reducing SCC linear scores throughout the first 6 mo of lactation; herein, we reported that udder health was not affected by ITMS because a reduction in clinical mastitis incidence was not observed. In the present study, we did not evaluate the effect of ITMS on SCC linear scores because of this information was not available in the farms' databases.

Although previous findings demonstrated that ITMS reduced uterine diseases, merely a tendency was reported herein. Machado et al. (2012) reported that ITMS reduced the presence of putative intrauterine pathogens in dairy cows 35 d after calving. Further, Machado et al. (2013) reported a lower incidence of clinical endometritis for cows that received ITMS according to the protocol performed in the present study. We also reported that ITMS numerically reduced the incidence of stillbirth, which has also been previously observed (Machado et al., 2013). Metritis and stillbirth parturition have been associated with impaired milk yield, poor reproductive performance, and culling in dairy cows (Bicalho et al., 2007, 2008; Dubuc et al., 2011; Wittrock et al., 2011). Because we did not detect positive effects of ITMS on reducing the risks of metritis and stillbirth parturition, no difference was detected in performance and survivability between the treatment groups. Likewise, Machado et al. (2013) observed that ITMS did not influence milk yield, reproductive outcomes, and culling of dairy cows. Additionally, Springman et al. (2018) and Vanegas et al. (2004) reported no beneficial effect of ITMS on reproduction outcomes in beef heifers and dairy cows, respectively.

Herein, we observed that ITMS improved PMNL function in the early postpartum period. Immune dys-

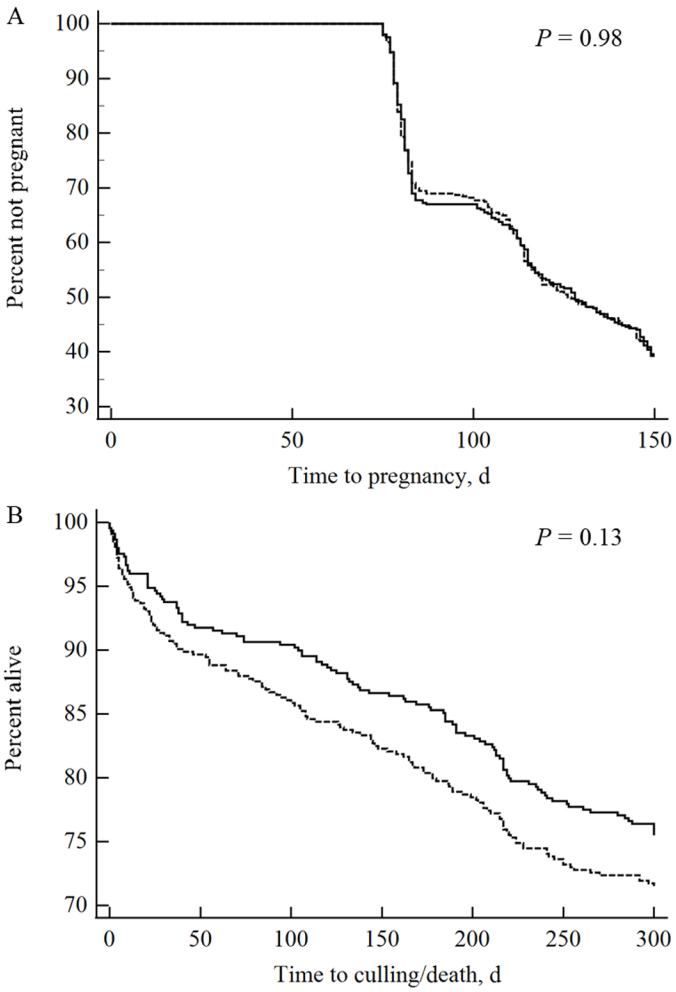

Figure 2. Kaplan-Meier survival analysis of (A) calving-to-conception interval and (B) days to culling or death for injectable trace mineral–supplemented (ITMS) cows [received 7 mL of subcutaneous injections of 15 mg/mL Cu, 5 mg/mL Se, 60 mg/mL Zn, and 10 mg/mL Mn (Multimin 90, Multimin North America) at 208 ± 3 d of gestation, 260 ± 3 d of gestation, and at 35 ± 3 DIM; dashed line] and control cows (solid line).

regulation during the postpartum period is thought to be one of the major causes of increased incidence of uterine and udder diseases in dairy cows (Kim et al., 2005; Hammon et al., 2006; Sordillo, 2013). However, in the present study, the greater proportions of PMNL performing phagocytosis and oxidative burst, coupled with improved oxidative burst intensity in ITMS-treated cows did not translate into improved health outcomes. Although Machado et al. (2014) reported that ITMS did not improve PMNL function at 10 DIM, others have observed that trace mineral supplementation improved immunity. Studies in beef and dairy cattle have demonstrated the benefit of using ITMS concurrently with bovine respiratory disease vaccines on humoral and cell-mediated immune responses (Palomares et al., 2016; Roberts et al., 2016; Bittar et al., 2018, 2020). Additionally, preweaning calves that received ITMS at 3 d of life had increased PMNL phagocytic activity at 14 d of life (Teixeira et al., 2014). The positive effect of trace mineral supplementation on PMNL activity may be linked to trace mineral action on intracellular enzymatic modulation, such as cytosolic superoxide dismutase (Cu- and Zn-dependent) and mitochondrial superoxide dismutase (Mn- and Zn-dependent; Suttle, 2010; Sordillo, 2016), but these variables were not evaluated herein, and more research is needed to test these hypotheses.

Additionally, the results of the current study suggested that trace mineral supplementation resulted in a more robust innate immune system in the early postpartum period. Initial steps in the recruitment process of PMNL to the infection sites involves rolling and attachment to the endothelium near the inflammation site. This process is dependent on the interaction between L-selectin protein adhesion receptors on the PMNL surface and its ligand on endothelial cells. Hence, we suggest that the earlier and greater neu-

Table 4. Effect of treatment on hematology, antioxidant enzymes, and haptoglobin (Hp)

	Treatment[2]			*P*-value		
Variable[1]	Control	ITMS	SEM	Treatment	Day[3]	Interaction
TLC, cells $\times 10^6$/mL	10.8	11.3	0.316	0.295	<0.0001	0.308
Monocytes, cells $\times 10^6$/mL	2.22	2.23	0.087	0.980	0.02	0.199
Neutrophil, cells $\times 10^6$/mL	1.84	2.04	0.134	0.273	<0.0001	0.463
Lymphocytes, cells $\times 10^6$/mL	6.99	7.15	0.324	0.692	<0.0001	0.506
Neutrophil:lymphocyte	0.39	0.49	0.033	0.039	0.02	0.869
GPx, nmol/min per mL	125.2	125.5	4.790	0.966	<0.0001	0.566
SOD, U/mL	3.52	3.63	0.076	0.320	<0.0001	0.249
Hp, mg/mL	139.9	122.7	9.682	0.210	<0.0001	0.735

[1]TLC = total leukocyte count; GPx = glutathione peroxidase; SOD = superoxide dismutase.

[2]Control cows (n = 74) received no trace mineral injection; ITMS cows (n = 68) received 7-mL subcutaneous injections of 15 mg/mL Cu, 5 mg/mL Se, 60 mg/mL Zn, and 10 mg/mL Mn (Multimin 90, Multimin North America) at dry-off (208 ± 3 d of gestation), 260 ± 3 d of gestation, and at 35 ± 3 DIM.

[3]Days relative to calving (−72, 3, 7, 10, 35 d).

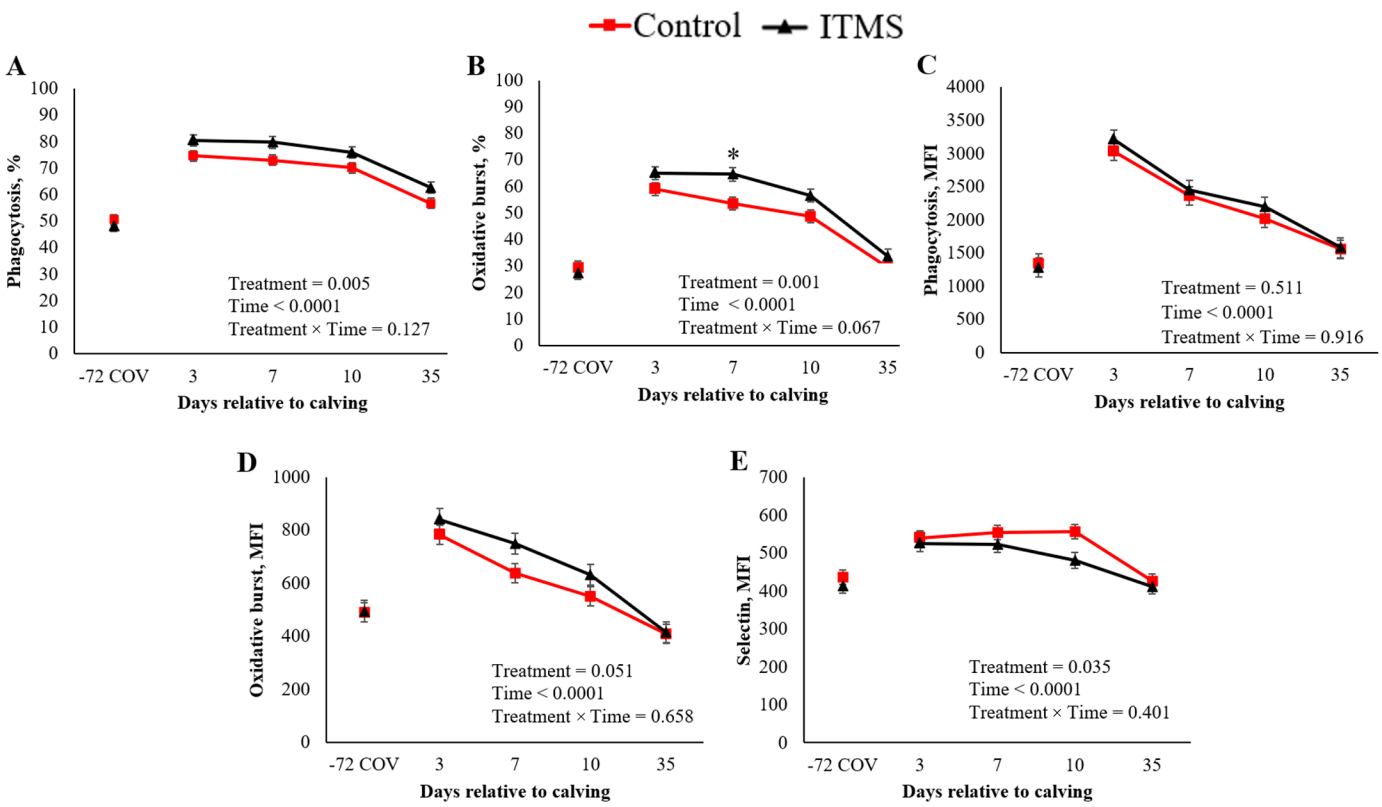

Figure 3. The effect of treatment on percentage of polymorphonuclear leukocytes (PMNL) that performed phagocytosis (panel A), percentage of PMNL that performed oxidative burst (panel B); phagocytosis mean fluorescence intensity (MFI; panel C), oxidative burst MFI (panel D), and L-selectin MFI (panel E). Control cows (n = 74) received no trace mineral injection; injectable trace mineral–supplemented ITMS cows (n = 68) received 7-mL subcutaneous injections of 15 mg/mL Cu, 5 mg/mL Se, 60 mg/mL Zn, and 10 mg/mL Mn (Multimin 90, Multimin North America) at dry-off (208 ± 3 d of gestation), 260 ± 3 d of gestation, and at 35 ± 3 DIM. COV = covariate (values at enrollment). Error bars represent SEM.*Indicates $P \leq 0.05$.

trophils activation detected in ITMS cows could have resulted in earlier regulation of inflammation, which led to a reduced expression of L-selectin on PMNL surface from ITMS-treated cows. The source of trace minerals fed to cattle seems to influence the expression of L-selectin. For instance, Jacometo et al. (2015) indicated that calves from dams fed organic trace minerals (Zn, Cu, Mn, and Co) during the last 30 d of gestation had lower L-selectin expression compared with calves from dams fed trace minerals from inorganic sources. Greater neutrophil-to-lymphocyte ratio was observed among the ITMS cows. Generally, neutrophil-to-lymphocyte ratio has been associated with chronic and acute inflammatory processes due to margination, redistribution, and accelerated apoptosis of lymphocytes (Zahorec, 2001). In the context of the other results observed herein, greater neutrophil-to-lymphocyte ratio among ITMS cows could represent greater inflammatory potential throughout the first days postpartum, which could have contributed to greater capacity to eliminate the uterine pathogens. Guan et al. (2020) reported that there is a positive association between inflammatory cytokines and neutrophil-to-lymphocyte ratio during the development of acquired immunity, leading to improved responsiveness of the immune system against pathogens. Additionally, because PMNL from ITMS cows were more active in the postpartum period, it is possible that PMNL from these cows stayed in the bloodstream longer due to the reduced L-selectin expression detected herein, which could be another explanation for the altered neutrophil-to-lymphocyte ratio. On the other hand, an overly robust inflammatory response during the transition period may lead to extensive damage to host tissues (Sordillo, 2016). However, we did not detect any negative effect of greater inflammatory potential of ITMS cows on health, milk yield, reproductive performance, and survivability.

In the present study, the lack of effect of ITMS in stillbirth may be a result of the inefficacy of ITMS to increase the activity of the antioxidant enzymes GPx and SOD. We initially hypothesized that potential benefits of ITMS on reducing stillbirth could be due to improved overall antioxidant capacity in the last month of lactation (Machado et al., 2013, 2014). In sows, high

total antioxidant capacity has been associated with a low incidence of stillbirth (Wang et al., 2019). Sciorsci et al. (2020) showed that blood ROS concentration undergoes an expressive increase from 180 to 210 d of pregnancy onwards. These ROS may compromise the physiology of the fetal-maternal unit, making it more susceptible to bacterial infection. Murray et al. (2008) reported that nutritional degenerative myopathy due to low uptake of Se and vitamin E was associated with myocardial necrosis found in stillborn fetuses. The authors explain that the Se-vitamin E complex, which increases GPx concentration, helps to protect the fetus and placenta against external ROS damage. However, it is important to highlight that we did not observe the effect of ITMS on GPx and SOD concentration and on stillbirth. Likewise, Bicalho et al. (2014) did not observe differences in serum SOD and GPx concentrations. On the other hand, Machado et al. (2014) detected higher blood SOD concentration in dairy cows during the transition period and subsequent lactation when supplemented with injectable trace minerals. Bernabucci et al. (2002) suggested that erythrocyte markers are a more appropriate and sensitive model to assess the oxidative status of moderate heat-stressed transition dairy cows than plasma markers. Further, Abuelo et al. (2013) considered that the concentration of anti- and pro-oxidants separately is not a good indicator of oxidative stress because it is the imbalance between them that defines the oxidative stress. Therefore, as we merely investigated the effect of ITMS on GPx and SOD activities of heat-stressed transition dairy cows, it is possible that we were not able to assess the real antioxidant capacity of the animals because trace minerals supplementation may act at other levels of the antioxidant network. In addition, as a limitation of this study, we did not assess any biological marker that could confirm the direct effect of elevated THI conditions on oxidative stress status of transition dairy cows.

The objectives of this study were to evaluate the effect of ITMS on the health, performance, postpartum immunity, and antioxidative status in dairy cows undergoing their transition period in an environment with THI >68. The meteorological analysis presented in Figure 1 demonstrates that THI was greater than 68 for most days when study cows were undergoing their transition period (3 wk before and 3 wk after calving). Additionally, all cows were housed in dry-lot pens during the dry period without fans and soaking lines, being more exposed to environmental changes in the weeks before calving. The THI has been widely used as an indicator of heat stress in dairy cows, and thus we believe that our study cows experienced heat-stress conditions during the transition period. However, the findings presented herein should be interpreted with care because we did not collect the physiological parameters for assessing heat stress (e.g., rectal temperature and respiration rate). According to Hammami et al. (2013), there are limitations to the use of THI to characterize heat stress. For instance, THI is an empirical representation of heat stress that does not assume the individual response of the animals to different environmental stressors. Moreover, the absence of other environmental factors in the THI equation, such as solar radiation and wind speed, are other limitations to this measure. Another study limitation could be the misclassification of diseases when using farm records, although the randomization procedure minimizes different distribution of this phenomenon by treatment groups.

CONCLUSIONS

Injectable trace mineral supplement containing Cu, Se, Zn, and Mn at dry-off (208 ± 3 d of gestation), 260 ± 3 d of gestation, and at 35 ± 3 DIM did not improve health, milk yield, reproductive performance, survivability, and the concentration of serum GPx and SOD concentrations of dairy cows undergoing the transition period in THI >68. However, our findings suggest that ITMS improved innate immunity during the early postpartum period, but this improvement in innate immunity response did not lead to improved health, as we only observed numerical changes in the incidence of metritis and stillbirth.

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