Translational Animal Science, 2024, **8**, txae028
https://doi.org/10.1093/tas/txae028
Advance access publication 2 March 2024
Immunology

Effect of exogenous melatonin on the cellular response of Holstein heifer calves during vaccination

Allie E. Martin,[†] Vinicius S. Machado,[‡] Ryan J. Rathmann[†], and Whitney L. Crossland[†,1]

[†]Department of Animal and Food Sciences, Texas Tech University, Lubbock, TX 79409, USA
[‡]Department of Veterinary Sciences, Texas Tech University, Lubbock, TX 79409, USA
[1]Corresponding author: whitney.crossland@ttu.edu

ABSTRACT

Despite rigorous vaccination protocols, calf morbidity is the primary contributor to economic loss in the calf sector of the dairy industry. Melatonin has modulated immune response in other mature animal species. We hypothesized that exogenous melatonin may improve the cellular response to vaccination in dairy calves. Our objective was to evaluate the effect of exogenous melatonin on polymorphonuclear leukocyte (**PMN**) function in Holstein heifer calves during immunization. Sixty neonatal Holstein heifers were enrolled by birth cohort (block) and randomized to one of four treatments: control (**CON**), vaccination of 0.5 mg ovalbumin on days 0 and 21 (**VAC)**, implantation of 24 mg melatonin on day 0 (**MEL**), or both melatonin and vaccine treatments (**MVAC**). Jugular blood was collected on days 0, 21, 42, and 63 to measure circulating melatonin, anti-ovalbumin immunoglobulin-G, and PMN function. Calves implanted with melatonin had greater circulating melatonin than non-implanted on day 21 ($P < 0.01$). Anti-ovalbumin IgG was greater for vaccinated than non-vaccinated calves ($P < 0.01$). Anti-ovalbumin IgG was greater for MVAC than VAC calves on day 63. Percent of cells and mean florescence intensity of cells performing oxidative burst decreased from day 0 to day 63 ($P < 0.01$) but were not affected by treatment ($P \geq 0.26$). There was a tendency ($P = 0.10$) for an interaction of melatonin, vaccination, and day for the mean florescence intensity of cells performing phagocytosis where MVAC was greater than all other treatments on d 42. Exogenous melatonin may alter PMN function of calves during vaccination. Further research is needed to define the effect of melatonin on development of antigen-specific IgG during programmed vaccination protocols.

LAY SUMMARY

New-born dairy calves are susceptible to disease due to an immature immune system. They primarily depend on their innate immune functions until acquired immunity is established through vaccination. Supplemental melatonin has been shown to improve circulating immune cells of adult animals during vaccination, but it has not been investigated in neonates. Using a mock antigen, we observed that when calves received both the antigen and melatonin, a greater percent of immune cells were active than vaccination alone. This has important implications for health management strategies because it occurs at a time when calves are at greatest risk of disease onset. There is potential that supplemental melatonin may bolster the immune system at critical times of development.

Key words: immunity, melatonin, Holstein, calves, cellular, humoral

INTRODUCTION

Despite best practices in colostrum management, biosecurity, and vaccination protocols, dairy calves remain susceptible to morbidity and mortality during the pre-weaning period. As part of the National Animal Health Monitoring System's Dairy 2014 study, Urie et al. (2018) reported a 5.0% mortality rate and 33.8% morbidity rate for preweaned heifers on U.S. dairy farms, with most morbid calves receiving at least one antibiotic treatment. As concerns of antimicrobial resistance increase, judicious use of antibiotics is a national priority. Consequently, other strategies are needed to aid in the prevention and treatment of disease, and bolster health during critical production periods.

The neonatal calf relies heavily on the innate polymorphonuclear leukocyte (**PMN**) function against invading pathogens (Chase et al., 2008). At birth the functionality of neutrophils is low but the passive transfer of antibodies through colostrum is known to rapidly increase phagocytic capacity of PMN (Menge et al., 1998). Failure of passive transfer has therefore been associated with greater morbidity in preweaned calves (Stilwell and Carvalho, 2011). Still, even calves with successful passive transfer may be susceptible to disease due to a gap in protection as they transition from maternal acquired immunity to vaccine acquired immunity (Chase et al., 2008), which may not be fully realized until well after weaning. This increases the responsibility of the innate response for pathogen clearance during this vulnerable time frame. Moreover, this susceptibility window is variable among calves and is antigen dependent (Fulton et al., 2004). Therefore, strategies which bolster the innate immune response during the preweaning period would be of value to the dairy industry. Human and murine models have indicated for decades that both endogenous and exogenous melatonin induces innate immunomodulation (Maestroni et al., 1986; Fraschini et al., 1990; Currier et al., 2000; Yu et al., 2000; Maestroni, 2001). In mice, melatonin has been shown to

Received September 8, 2023 Accepted February 29, 2024.

support the production of natural killer cells and monocytes, and inhibit apoptosis of B cells (Currier et al., 2000; Yu et al., 2000; Maestroni, 2001). Activation of G-protein-coupled cell membrane melatonin receptors in T-helper cells leads to the release of T-helper cell type 1 cytokines (Maestroni, 2001). Melatonin binds to either membrane or nuclear receptors in antigen or cytokine-activated peripheral T-helper cells, peripheral macrophages, and bone marrow Th cells, leading to increased production of cytokines and melatonin-induced opioids (Maestroni, 2001). More recently, the neutrophil expression of MT2 (melatonin receptor 2) was shown to be upregulated in mice following bacterial infection (Xu et al., 2019). Xu et al. (2019) also indicated that melatonin treated neutrophils had greater bacterial clearance compared to non-melatonin treated neutrophils. The pharmacological use of melatonin to establish immunity in livestock species has been previously investigated but is very limited. In ewes, exogenous melatonin acted as an adjuvant and increased antibody titer to antigens implicated in foot rot (Regodón et al., 2009). When administered as either an injection or implant, melatonin increased antibody titers as well as T- and B-cell ratios in ewes (Ramos et al., 2018). While these outcomes clearly demonstrate immunomodulation, these experiments were accomplished in mature animals with mature immune systems. This leaves a critical gap in knowledge on the strategic use of melatonin in livestock with immature immune systems.

We hypothesized that the application of melatonin could enhance the immune response of calves during crucial immunological events. Therefore, our objective was to investigate the effect of an exogenous melatonin implant on the PMN function of neonatal Holstein heifer calves and its interaction with vaccine stimulation using ovalbumin as a model antigen.

MATERIALS AND METHODS

The Texas Tech University Institutional Animal Care and Use Committee approved all experimental protocols (approval number #21004-01). Calves were housed and cared for at a commercial dairy in Amherst, TX as described in a subsequent section.

Sample Size Calculation

Sample size calculation was undertaken in JMP PRO 16 (SAS Institute Inc., Cary, NC). To determine differences between melatonin-implanted vs. control groups, we assumed that the concentration of melatonin would increase 1-fold from baseline, pre-implant values (25.6 vs. 51.2 pg/mL) based on data by Wang et al. (2019) for daytime circulating melatonin in dairy cows. Considering a significance level of $\alpha = 0.05$ and a power of 80%, 4 implanted and 4 non-implanted would need to be enrolled. To determine differences between vaccinated vs. non-vaccinated groups, based on previously reported data (Rivera et al., 2002), we assumed that the optical density (OD) of anti-ovalbumin IgG would increase at least 1 OD unit above prevaccinated values. Considering a significance level of $\alpha = 0.05$ and a power of 80%, 46 calves would need to be enrolled (23 each in vaccinated and non-vaccinated groups). To determine differences in the mean fluorescence intensity (MFI) of oxidative burst among all four treatment groups, we assumed that MFI would move at least one standard deviation away from the d 0 mean. With a significance level of $\alpha = 0.05$ and a power of 80%, 40 calves would need to be enrolled (10 per treatment). To account for attrition, a sample of 60 calves (15 per treatment) was taken. With this sample size, we were able to detect an anti-ovalbumin IgG OD difference between the groups of 0.87 OD with standard deviation of 1.86 OD, considering $\alpha = 0.05$ and a power of 80%.

Ovalbumin Vaccine Preparation

Ovalbumin vaccination was prepared by dissolving 0.5 mg of ovalbumin (InvivoGen, San Diego, CA) in 0.5 mL 0.1 M PBS and emulsified in 0.5 mg of adjuvant Quil-A (InvivoGen) that was dissolved in 0.5 mL of PBS (Magalhães et al., 2008). The mixture was stored at –20 °C and thawed at the time of treatment.

Animals, Housing, and Treatment Allocation

From February to May of 2021, Holstein heifer calves were used for 63 d in a blocked randomized clinical trial design with a 2 × 2 factorial arrangement of treatments, where calf was the experimental unit and block was the birth cohort at the time of enrollment. Calves were housed outdoors (avg low = 7.8 °C, avg high = 24 °C) in individual calf hutches (223.52 cm × 128 cm × 140 cm) at a commercial dairy in the Texas panhandle and were bottle fed 3 L of plasma protein milk replacer twice a day with free access to water and a pelleted starter grain. At 0 day of age heifers received 2 mL of a three-way MLV vaccine containing IBR, PI3, and BRSV (Inforce3, Zoetis, Parsippany, NJ), and a bolus of antibodies for Bovine Coronavirus and *Escherichia coli* (First Defense, ImmuCell Corporation, Portland, ME). Calves were enrolled as a cohort (blocking factor) at 3 to 5 days of age (day 0 of trial). Within 72 h of birth, peripheral blood was drawn from the jugular vein to measure total serum protein (**TSP**). Calves were assigned randomly to treatment (n = 15 total per treatment) within block. Treatment factors consisted of a 2-series ovalbumin immunization or no immunization, and a melatonin implant or no implant, resulting in 4 treatment groups: control (**CON**), vaccine only (**VAC**), implant only (**MEL**), and implant + vaccine (**MVAC**). Blood samples were drawn on days 0, 21, 42, and 63 at approximately 1 h after sunrise. Heifers in the VAC and MVAC groups received a 1-mL ovalbumin vaccination administered subcutaneously in the triangle of the neck on days 0 and 21, after blood collection. Calves receiving melatonin (MEL and MVAC) were given 24 mg of exogenous melatonin in a sub-dermal, slow-release implant (Dermatonin; Melatek LLC, Prairie du Sac, WI; recommended 24 mg for large animals) in the posterior middle one-third of the ear between cartilage ribs on day 0. At 28 days of age (between trial days 23 and 25 dependent on calf birthdate), heifers received 2 mL of a three-way MLV vaccine containing IBR, PI3, and BRSV (Nasalgen3, Intervet/Merck Animal Health).

Blood Collection

Between days –1 and –3, blood (10 mL) was drawn from the jugular vein into an evacuated whole blood tube, centrifuged at 1,500 × g for 5 min at 4 °C, and analyzed for TSP using a handheld refractometer (Atago, Japan). On day 0, jugular blood was collected into two evacuated tubes (Vacutainer, Becton, Dickson, and Co., Franklin Lakes, NJ) containing 158 USP sodium heparin or 18 mg K2 EDTA. The heparinized blood was used to assess PMN by measuring phagocytic and oxidative burst activity. To preserve cell activity, heparinized blood was transported at ambient temperature (Sellers et al., 2013), whereas coagulated blood was transported on ice

to the laboratory. Blood tubes containing K2 EDTA were centrifuged at 2,000 × *g* for 15 min at 4 °C, and plasma was aliquoted in triplicate and frozen at −80 °C for downstream analyses of neutralizing anti-ovalbumin IgG and melatonin concentrations.

Melatonin ELISA

Blood samples used to measure circulating melatonin were collected approximately 1 h after sunrise to minimize interference of naturally secreted melatonin. Serum melatonin concentrations were measured using a commercial ELISA kit (Eagle Biosciences Inc., Amherst, NH) according to manufacturer's instructions. Briefly, 25 µL of calibrators (0, 3, 10, 30, 100, and 300 pg/mL), controls, or samples were added in duplicate to a 96-well microplate coated with goat anti-rabbit globulin. Subsequently, 50 µL of melatonin HRP conjugate and melatonin EIA rabbit monoclonal antibody was added to each well and allowed to incubate for 120 min on an orbital shaker at 550 rpm at room temperature. Plates were washed with 300 µL of wash solution provided in the kit. The color reaction was developed with 50 µL of tetramethylbenzidine plus hydrogen peroxide for 30 min at room temperature and stopped with 50 µL of a diluted sulfuric and hydrochloric acid solution. Plates were read at 450 nm on a EPOCH2 microplate reader within 10 min of stopping the reaction. Concentrations of controls and samples were determined by interpolation using a 4-parameter sigmoid fit software (MyAssay). The intra- and inter-assay CV were 15.7% and 26.3%, respectively. The source of variation was greatest among serum with less than 50 pg/mL (CV =23.9%). The CV of serum melatonin among calves with greater than 50 pg/mL was 8.9%. Validation using kit controls were also found to be more variable with the low working concentration (Kit control 1 = 6 pg/mL; CV = 32%) versus the high concentration (Kit control 2= 80 pg/mL; CV = 4.0%). Due to the relative expense and difficulty of the assay a random sub-set of 8 heifers from each treatment group (n = 32) were used in the final lab analysis. Moreover, the absolute values of circulating melatonin were not necessarily of interest, rather, they were measured to ensure melatonin implant did indeed cause elevated levels of circulating melatonin above normal daytime levels. We acknowledge pipetting skill may be to blame with an especially low working concentration.

Anti-ovalbumin IgG ELISA

Ovalbumin-specific antibodies were analyzed using an indirect ELISA (Ballou and DePeters, 2008; Silva et al., 2015; Belli et al., 2018). A 96-well plate was coated with 100 µL of a 44.04-mM carbonate and 6.04-mM bicarbonate buffer containing 1.4 g/L of ovalbumin and incubated for 24 h at 4 °C. Wells were washed three times with 300 µL of PBS containing 0.05% Tween (PBST). To block non-specific binding sites, 300 µL of PBS containing 4% bovine serum albumin were added to each well and incubated at room temperature for 2 h, after which plates were washed with PBST three times. Next, 200 µL of diluted serum (1:1,000 in PBST) were added to each well. Following a 2-h incubation period, plates were washed as previously described, and 100 µL of PBST with rabbit anti-bovine IgG conjugated to alkaline phosphatase diluted (1:10,000) were added. Plates incubated for 1 h and were washed three times with PBST. After the addition of 50 µL of 3,3',5,5'-tetramethylbenzidine (Cat# 34028, ThermoFisher Scientific, Waltham, MA) to each well, plates were incubated in the absence of light for 15 min. To stop the reaction, 50 µL of 0.16 M sulfuric acid were added, and plates were read immediately at 450 nm on a EPCOH2 microplate reader (BioTek Instruments, Inc., Wincoski, Vermont). The optical density recorded was indicative of the amount of antibody bound to the antigen in the wells. Samples were analyzed in duplicate and to account for non-specific binding, one additional well per sample was blank. Blank wells were not coated with ovalbumin. The working concentration range was 0.1 to 10 ng/mL and coefficient of variation was 5.4% and 8.7% for intra- and inter-assay, respectively. Since there is no clinical significance for determining protective titers of anti-ovalbumin, data were reported as the average optical density (**OD**) as proof of concept for augmented humoral response (Ballou et al., 2008; Silva et al., 2015; Belli et al., 2018). Average of the duplicates is reported with the OD of the corresponding blank subtracted.

PMN Function Analysis

The oxidative burst and phagocytic capacities of PMN were analyzed via flow cytometry. Sample preparation followed the procedure described by Silva et al. (2022) with minor adaptations. Briefly,100 µL of heparinized whole-blood transferred to a low-adhesion microcentrifuge tube and incubated in an ice bath for 15 min. Next, 20 µL of propidium iodide labeled *E. coli* (Invitrogen, Carlsbad, CA) with a concentration of 109 CFU/mL and 20 µL of 100 µM dihydrorhodamine solution were added to each sample and incubated in a 38.5 °C water bath for exactly 10 min, while negative controls incubated in an ice bath. All samples were then placed in an ice bath for 15 min. Red blood cells were hypotonically lysed by the addition of 800 µL of ice cold H_2O. Thirty seconds later, 200 µL of 5X PBS was added to stop lysis. Samples were centrifuged for 5 min at 1,200 × *g* and blood cells were aspirated. Remaining cells were lysed once again, and 1 mL of PBS was added to the remaining leukocytes. Dual-color flow cytometry was performed using an Attune flow cytometer (ThermoFisher Scientific, Denver, CO). The PMN populations were gated based on forward and side scatter plots. The optical filters BL1 (excited by a 488-nm laser on a 530/30 filter) and BL3 (excited by a 488-nm laser on a 695/40 filter) were used to determine the MFI and percentage of PMN that performed oxidative burst and phagocytosis, respectively. Negative and positive signals on the BL3 and BL1 scatterplot were acquired by use of negative controls.

Statistical Analyses

Data were analyzed in R version 4.1.0. Animal served as experimental unit. A three-way ANOVA was used where melatonin, vaccination, and day were fixed effects and cohort (block) as a random effect; day was repeated, and the subject of the repeated statement was animal. The Lmer function (Kunzetsova et al., 2017) was used to build linear mixed effect models. A two-way ANOVA was used when three-way interactions were not present, and contrasts were investigated within day for VAC vs MVAC to determine whether melatonin affected cellular and humoral response more than VAC alone. The Tukey adjustment method was used to conduct pairwise comparisons at individual time points. Total serum protein was evaluated as a covariate in all models and

Table 1. Descriptive statistics of assays measured on day 0[1]

Item	Mean	SD	CV, %	Minimum	Maximum
Total serum protein, g/dL[2]	5.8	0.72	12.4	4.20	7.40
Melatonin, pg/mL	25.9	15.8	61.2	6.56	81.6
Anti-ovalbumin IgG, OD[3]	0.107	0.020	6.82	−0.06	0.52
Oxidative burst[4]					
%	46.9	13.7	29.2	8.38	73.6
MFI	578	190	32.9	190	1,159
Phagocytosis[4]					
%	53.5	12.6	23.6	17.1	78.4
MFI	537	118	22.1	347	912

[1]Statistics across treatments of variables measured on day 0; $n = 60$, except melatonin subsampled at $n = 32$ (8/treatment). Except for total serum protein, measurements were taken at 3 to 5 days of age.
[2]Total serum protein reference of 5.2 g/dL considered successful passive transfer. 83.33% of calves had successful passive transfer. Measured within 48 h of birth.
[3]OD = optical density.
[4]Percentage of cells performing the given function and MFI = mean florescence intensity (magnitude).

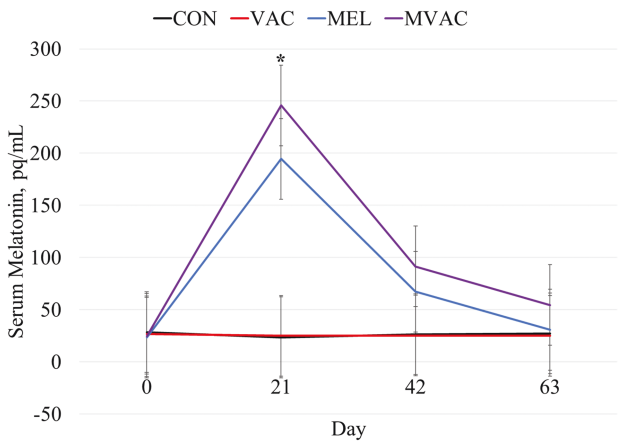

Figure 1. Effect of melatonin implant on circulating melatonin in Holstein heifer calves. Values are mean serum melatonin concentration determined by a commercial assay of heifer calves receiving no treatment (CON; $n = 8$), vaccinated with ovalbumin (VAC; $n = 8$) on days 0 and 21, implanted with 24 mg of melatonin (MEL; $n = 8$) on day 0, or received both VAC and MEL treatments (MVAC; $n = 8$). Day 0 values are measurments prior to implantation. Effects of melatonin, vaccination, day, and all two- and three-way interactions were evaluated. Vertical bars represent SEM. A melatonin by day interaction occurred ($P \leq 0.01$) where serum melatonin of implanted heifers was increased by day 21, but decreased and was no longer different than non-implanted heifer calves by day 42.

excluded if $P \geq 0.10$. Results were considered statistically significant when $P \leq 0.05$. Tendencies were identified when $P \leq 0.10$, but > 0.05.

RESULTS

Descriptive Statistics

As noted previously, 60 calves were enrolled in the study. Descriptive statistics including TSP, PMN activities, and melatonin concentrations on day 0 are reported in Table 1. Mean ± SD total serum protein for Holstein heifers at 3 to 5 d of age was 5.8 g/dL[2]. Circulating melatonin at 1 h after sunrise was 25.9 ± 15.8 pg/mL and anti-ovalbumin IgG, OD was 0.107 ± 0.02% transmission, indicating no previous IgG to ovalbumin was present. The percentage of cells performing oxidative burst was 46.9 ± 13.7% and mean florescence intensity (**MFI**) was 578 ± 190. The percentage of cells performing phagocytosis was 53.5 ± 12.6% and MFI was 537 ± 118.

Circulating Melatonin

There was no three-way interaction of melatonin, vaccine, and day on circulating melatonin. As expected, there was an interaction of melatonin and day ($P < 0.01$; Figure 1). Heifers implanted with melatonin had an 806.6% increase in circulating serum melatonin concentrations by day 21 when compared with non-implanted heifers but were no longer different by day 42. Since the implant was designed for smaller mammals, it was not unexpected for the lifespan of the implant to be shorter than the 90-d claim. Still the treatment application elicited the desired response of greater circulating melatonin during the critical window of vaccination (days 0 and 21).

Anti-ovalbumin IgG

There was no three-way interaction of melatonin, vaccine, and day on anti-ovalbumin IgG ($P = 0.86$). As expected, there was a vaccine by day interaction for IgG ($P < 0.01$; Figure 2a), where vaccinated calves had greater anti-ovalbumin IgG on days 21, 42, and 63 compared with unvaccinated calves. Interestingly, within the linear contrast of VAC versus MVAC (Figure 2b), least squared means tended to differ on day 42 ($P = 0.07$) and were greater for MVAC than VAC on day 63 (2.71 vs 2.25 OD, respectively; $P = 0.04$). Despite this interesting outcome, authors felt conservative about its interpretation as light transmission is quite low at OD greater than 1. Replication of this particular outcome is needed as it was not the initial aim of this experiment.

PMN Leukocyte Function

There were no three- or two-way interactions for the percent of leukocytes performing oxidative burst (Figure 3a), the MFI of oxidative burst (Figure 3b) or the percent of leukocytes performing phagocytosis (Figure 3c). Percent of leukocytes performing oxidative burst was affected by day

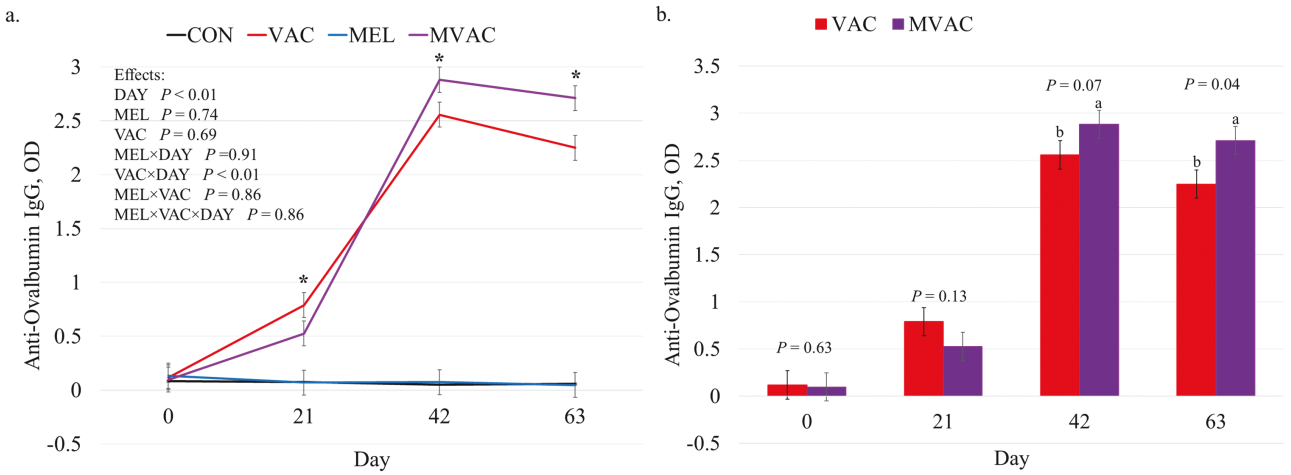

Figure 2. (a) Effect of ovalbumin vaccination on days 0 and 21 on anti-ovalbumin IgG in plasma (OD, optical density). Values are mean anti-ovalbumin in vaccinated heifer calves receiving no treatment (CON; $n = 15$), vaccinated with ovalbumin (VAC; $n = 15$) on days 0 and 21, implanted with 24 mg of melatonin (MEL; $n = 15$) on day 0, or received both VAC and MEL treatments (MVAC; $n = 15$). Day 0 values are measurements prior to vaccination. Effects of melatonin, vaccination, day, and interactions were evaluated. Vertical bars represent SEM. There was a vaccination by day interaction ($P < 0.01$) and denoted with asterisk where vaccinated calves had increased anti-ovalbumin IgG by day 21 and increased toward day 42 but non-vaccinated calves did not change. The mock antigen was successful at initiating a humoral response. (b) Linear contrast of two way-ANOVA of melatonin and vaccine. Values are means of ovalbumin vaccinated calves implanted with 24 mg of melatonin (MVAC; $n = 15$) or vaccine only (VAC; $n = 15$). Anti-ovalbumin was not different between VAC an MVAC calves on days 0 and 21. Within day 42, there was a tendency ($P = 0.07$) for calves who received a melatonin implant in addition to vaccination to have greater OD of IgG, and this was significantly different ($P = 0.04$) on day 63.

($P < 0.01$), being greater on days 0, 21, and 42, but lesser on day 63. The mean fluorescence intensity of cells performing oxidative burst was also affected by day ($P < 0.01$) which decreased successively from days 0 to 63. Percent of leukocytes performing phagocytosis was affected by day ($P < 0.01$) where days 21 and 42 were greater than days 0 and 63. There was a tendency ($P = 0.10$) for a three-way interaction of melatonin, vaccination, and day on the mean fluorescence intensity of phagocytic cells (Figure 3c). The MFI of phagocytic cells increased for all treatments after day 0; however, heifer calves in the VAC treatment group peaked at day 21 versus CON, MEL, and MVAC peaked on day 42 before declining on day 63. Within day 42, phagocytic MFI of MVAC treated heifers was greater than the mean of other treatments (1,083 vs. mean of 794 MFI, $P < 0.01$).

DISCUSSION

Many studies have investigated the immunomodulatory effects and pathways of melatonin (Maestroni et al., 1986; Fraschini et al., 1990; Maestroni, 1995, 2001; Barjavel, et al., 1998; García-Mauriño, et al., 1999; Currier et al., 2000; Yu et al., 2000; Chase et al., 2008). Nonetheless, the effects of melatonin supplementation on innate and adaptive immune response in cattle are still unknown, and there has been no research in the most vulnerable population of calves with immature immune systems. Melatonin research in cattle has primarily focused on the effects of melatonin on uterine blood flow (Lemley et al., 2012; McCarty et al., 2018).

In the current study, melatonin alone did not augment PMN function but may have influenced the innate response to vaccination by increasing leukocyte phagocytosis efficiency and perhaps affected circulating antibodies. This preliminary outcome is consistent with the results of work in mature ruminants (Regodón et al., 2009), where melatonin implants increased antibody titers of ewes vaccinated against *Dichelobacter nodosus*. Based on extensive research on effects of melatonin in humans and mice, Maestroni (2001) proposed that melatonin could boost vaccines to intracellular pathogens by supporting the free radical scavenging properties of Th1 cells. In essence, the binding of melatonin to receptors of active T-helper cells might upregulate cytokine production. Although these specific responses of the adaptive immune system were not evaluated directly in the current experiment, we measured the production of neutralizing antibodies to ovalbumin (produced by the humoral response) to not only ensure the antigen elicited the desired response but also to determine any benefit of melatonin on the adaptive humoral response. The tendency for MVAC-treated calves to has greater IgG on day 42 might be linked to the previously mentioned upregulation of activated T cells to produce cytokines. Specifically, the upregulation of IL-2, which also stimulates the proliferation of T cells and IgG expressing B cells, and thus a re-enforcing loop of immune response to antigen. Notably, antibodies to ovalbumin and leukocyte phagocytosis activity were the greatest on day 42 for the MVAC-treated calves, whereas the VAC group was achieved on day 21. Perhaps this outcome is a result of antibody receptor-mediated phagocytosis through opsonization. Opsonins are molecules that coat bacterium in positively charged molecules to attract neutrophils to perform phagocytosis on the bacterium, and antibodies are the most effective opsonin (Tizard 2018). We therefore speculate that the increase in MFI was caused by antibody receptor-mediated phagocytosis triggered by the increase in circulating antibodies. Nonetheless, this does not fully explain why calves that received VAC did not also have greater phagocytic activity on day 42, while still presenting substantial ovalbumin IgG. Early work by Fraschini et al. (1990) indicated that melatonin may only be effective in situations where the immune system is already stimulated.

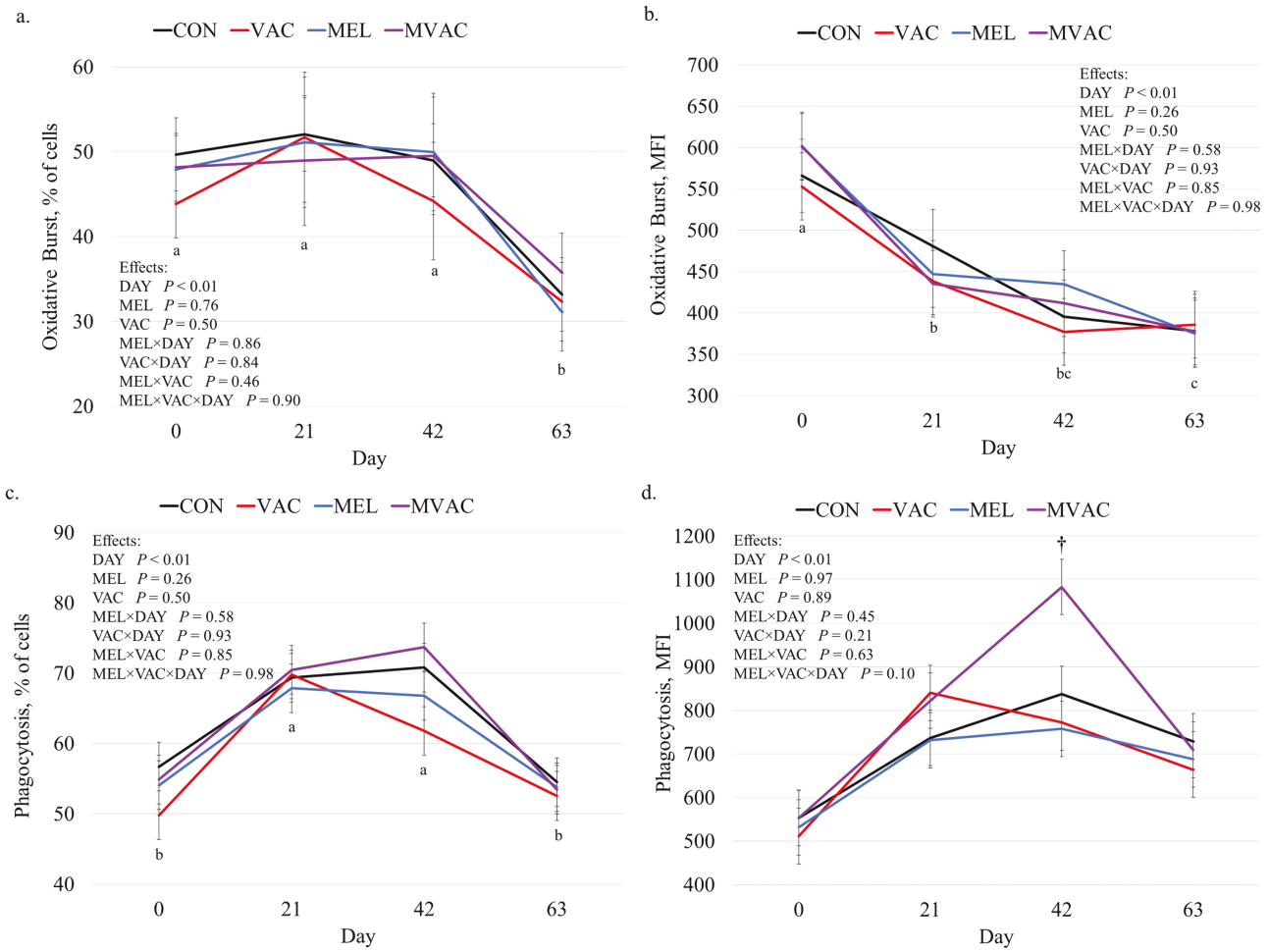

Figure 3. Measures of polymorphonuclear leukocyte function in calves receiving no treatment (CON), vaccinated with ovalbumin (VAC) on days 0 and 21, implanted with 24 mg of melatonin (MEL) on day 0, or received both VAC and MEL treatments (MVAC). Effects of melatonin, vaccination, day, and interactions were evaluated but only relevant *P*-values are provided. Vertical bars represent SEM. Within a panel differing superscripts (a-c) indicate an effect of day and means differed at $P \leq 0.05$ and † (Panel d) indicates a tendency $0.05 < P \leq 0.10$ for means to be different. (a) Percentage of neutrophils performing oxidative burst. Percentage of neutrophils performing oxidative burst was affected by day ($P < 0.01$) where means were greater for days 0, 21, 42, and lesser for day 63. (b) Mean fluorescence intensity (MFI) of oxidative burst. MFI was affected by day ($P < 0.01$) where means were greatest on day 0 and decreased by day 42. (c) Percentage of neutrophils performing phagocytosis. Percentage of neutrophils performing phagocytosis was affected by day ($P < 0.01$) where means were lesser for days 0 and 63 but greatest for days 21 and 42. (d) Mean fluorescence intensity of phagocytosis. MFI tended to be different ($P = 0.10$) where CON, MEL, MVAC treatments increased from days 0 to 42 and then decreased, while VAC increased from days 0 to 21 but decreased to day 42. Contrasts within day 42 reveal phagocytic MFI was greater ($P < 0.01$) for MVAC than VAC on day 42.

This is demonstrated by the more recent work by Xu et al. (2019), such that the increase in phagocytic capacity could be driven by the upregulation of melatonin receptor-2 expression in infected neutrophils. In Xu et al. (2019), this was mediated by neutrophil extracellular trap (**NET**) formation rather than phagocytosis. Production of NET, however, does not seem to be active in neonates (Yost et al., 2009), which indicates their reliance on effective phagocytosis. These principal findings could explain why there was no effect of MEL alone on PMN function, but when in the presence of an activated immune response by vaccination (MVAC), the greater circulating melatonin would reinforce the killing capacity of PMN leukocytes more than VAC alone, which was observed at day 42.

The application of these research outcomes for animal production settings warrants further investigation. Titer response as an accurate predictor of disease is either disease-specific or nonexistent (Martin and Bohac, 1986; Martin et al., 1990; Downey-Slinker et al., 2016). Further research is needed to determine whether an elevated titer response resulting from exogenous melatonin would lead to decreased morbidity or antibiotic intervention in challenge models. Yet it is encouraging to see data that suggests there is an improvement in the innate response, especially in at-risk groups of livestock like pre-weaned dairy calves. Clearly, melatonin plays a role in the cell-mediated cascade of events during an antigen-activated immune response. Outcomes of the current experiment prompt investigation into more strategic use of melatonin, and results of such work could lead to new management strategies for vaccination protocols. As Maestroni (2001) suggested over 20 yr ago, the implementation of melatonin could be highly translational across livestock species and especially important for high-stress events. A limitation of using the model antigen, ovalbumin, in neonatal calves is the lack of interference of maternal antibodies from colostrum that would typically be encountered with common bovine pathogens. Further research is needed to evaluate whether exogenous melatonin could impact the antibody

response to vaccines already on the market and whether improved immunity could translate to both short- and long-term improvements in clinical health of calves that are still developing an immune system.

Conclusions

Results of the current study demonstrate that administration of exogenous melatonin through slow-release implant could improve the innate immune response during vaccination in pre-weaned dairy heifer calves, particularly phagocytosis capacity. Continued research in this area is warranted to understand how melatonin may increase the IgG to specific antigens during a vaccination program. Due to the relative expense of implant technology, alternate application methods and dosage levels will need to be determined for industry adoption. These findings ultimately add to the literature that exogenous melatonin may improve innate immunity, vaccine responses, in the most vulnerable population of calves who are still developing an immune system, which has not previously been investigated.

Supplementary Data

Supplementary data are available at *Translational Animal Science* online.

Acknowledgments

We would like to thank Tim Cairns at Melatek LLC for the in-kind donation of the melatonin implants for research purposes.

Conflict of Interest

The authors have no real or perceived conflict of interest.

Literature Cited

Ballou, M. A., and E. J. DePeters. 2008. Supplementing milk replacer with omega-3 fatty acids from fish oil on immunocompetence and health of Jersey calves. J. Dairy Sci. 91:3488–3500. doi:10.3168/jds.2008-1017

Barjavel, M. J., Z. Mamdouh, N. Raghbate, and O. Bakouche. 1998. Differential expression of the melatonin receptor in human monocytes. J. Immunol. (Baltimore, MD: 1950). 160:1191–1197. doi:10.4049/jimmunol.160.3.1191.

Belli, A. L., R. B. Reis, A. Veronese, R. Moreira, K. Flanagan, J. Driver, C. D. Nelson, J. A. Clapper, M. A. Ballou, K. C. Jeong, et al. 2018. Effects of treatment of preweaning dairy calves with recombinant bovine somatotropin on immune responses and somatotropic axis. J. Dairy Sci. 101:6602–6615. doi:10.3168/jds.2017-13917

Chase, C. C., D. J. Hurley, and A. J. Reber. 2008. Neonatal immune development in the calf and its impact on vaccine response. Vet. Clin. North Am. Food Anim. Pract. 24:87–104. doi:10.1016/j.cvfa.2007.11.001

Currier, N. L., L. Z. Y. Sun, and S. C. Miller. 2000. Exogenous melatonin: quantitative enhancement in vivo of cells mediating non-specific immunity. J. Neuroimmunol. 104:101–108. doi:10.1016/s0165-5728(99)00271-4

Downey-Slinker, E. D., J. F. Ridpath, J. E. Sawyer, L. C. Skow, and A. D. Herring. 2016. Antibody titers to vaccination are not predictive of level of protection against a BVDV type 1b challenge in Bos indicus - Bos taurus steers. Vaccine 34:5053–5059. doi:10.1016/j.vaccine.2016.08.087

Fraschini, F., F. Scaglione, P. Franco, G. Demartini, V. Lucini, and B. Stankov. 1990. Correspondence and short communications melatonin and immunity. Acta Oncol. 29:775–775. doi:10.3109/02841869009092998

Fulton, R. W., R. E. Briggs, M. E. Payton, A. W. Confer, J. T. Saliki, J. F. Ridpath, L. J. Burge, and G. C. Duff. 2004. Maternally derived humoral immunity to bovine viral diarrhea virus (BVDV) 1a, BVDV1b, BVDV2, bovine herpesvirus-1, parainfluenza-3 virus bovine respiratory syncytial virus, Mannheimia haemolytica and Pasteurella multocida in beef calves, antibody decline by half-life studies and effect on response to vaccination. Vaccine 22:643–649. doi:10.1016/j.vaccine.2003.08.033

García-Mauriño, S., D. Pozo, A. Carrillo-Vico, J. R. Calvo, and J. M. Guerrero. 1999. Melatonin activates Th1 lymphocytes by increasing IL-12 production. Life Sci. 65:2143–2150. doi:10.1016/s0024-3205(99)00479-8

Kunzetsova, A., P. Brockhoff, and R. Christensen. 2017. lmerTest package: tests in linear mixed effect models. J Stat Softw. 82:1–26. doi:10.18637/jss.v082.i13.

Lemley, C. O., A. M. Meyer, L. E. Camacho, T. L. Neville, D. J. Newman, J. S. Caton, and K. A. Vonnahme. 2012. Melatonin supplementation alters uteroplacental hemodynamics and fetal development in an ovine model of intrauterine growth restriction. Am. J. Physiol. Regul. Integr. Comp. Physiol. 302:R454–R467. doi:10.1152/ajpregu.00407.2011

Maestroni, G. J. 1995. T-Helper-2 lymphocytes as a peripheral target of melatonin. J. Pineal Res. 18:84–89. doi:10.1111/j.1600-079x.1995.tb00144.x

Maestroni, G. J., A. Conti, and W. Pierpaoli. 1986. Role of the pineal gland in immunity: circadian synthesis and release of melatonin modulates the antibody response and antagonizes the immunosuppressive effect of corticosterone. J. Neuroimmunol. 13:19–30. doi:10.1016/0165-5728(86)90047-0

Maestroni, G. J. M. 2001. The immunotherapeutic potential of melatonin. Expert Opin Investig. Drugs 10:467–476. doi:10.1517/13543784.10.3.467

Magalhães, V. J. A., F. Susca, F. S. Lima, A. F. Branco, I. Yoon, and J. E. P. Santos. 2008. Effect of feeding yeast culture on performance, health, and immunocompetence of dairy calves. J. Dairy Sci. 91:1497–1509. doi:10.3168/jds.2007-0582

Martin, S. W., K. G. Bateman, P. E. Shewen, S. Rosendal, J. G. Bohac, and M. Thorburn. 1990. A group level analysis of the associations between antibodies to seven putative pathogens and respiratory disease and weight gain in Ontario feedlot calves. Can. J. Vet. Res. 54:337–342. https://pubmed.ncbi.nlm.nih.gov/2165846/.

Martin, S. W., and J. G. Bohac. 1986. The association between serological titers in infectious bovine rhinotracheitis virus, bovine virus diarrhea virus, parainfluenza-3 virus, respiratory syncytial virus and treatment for respiratory disease in Ontario feedlot calves. Can. J. Vet. Res. 50:351–358.

McCarty, K. J., M. P. Owen, C. G. Hart, R. C. Thompson, D. D. Burnett, E. H. King, R. M. Hopper, and C. O. Lemley. 2018. Effect of chronic melatonin supplementation during mid to late gestation on maternal uterine artery blood flow and subsequent development of male offspring in beef cattle. J. Anim. Sci. 96:5100–5111. doi:10.1093/jas/sky363

Menge, C., B. Neufeld, W. Hirt, N. Schmeer, R. Bauerfeind, G. Baljer, and L. H. Wieler. 1998. Compensation of preliminary blood phagocyte immaturity in the newborn calf. Vet. Immunol. Immunopathol. 62:309–321. doi:10.1016/s0165-2427(98)00109-3

Ramos, A., M. P. Míguez, S. Morgado, B. Sanchez-Correa, J. J. Gordillo, J. G. Casado, R. Tarazona, and S. Regodón. 2018. Melatonin enhances responsiveness to Dichelobacter nodosus vaccine in sheep and increases peripheral blood CD4 T lymphocytes and IgG-expressing B lymphocytes. Vet. Immunol. Immunopathol. 206:1–8. doi:10.1016/j.vetimm.2018.11.006

Regodón, S., A. Ramos, S. Morgado, R. Tarazona, P. Martín-Palomino, J. A. Rosado, and M. P. Míguez. 2009. Melatonin enhances the

immune response to vaccination against A1 and C strains of *Dichelobacter nodosus*. Vaccine 27:1566–1570. doi:10.1016/j.vaccine.2009.01.001

Rivera, J. D., G. C. Duff, M. L. Galyean, D. A. Walker, G. A. Nunnery. 2002. Effects of supplemental vitamin E on performance, health, and humoral immune response of beef cattle. *J. Anim. Sci*. 80:933–941. doi:10.2527/2002.804933x.

Sellers, M. D., L. E. Hulbert, and M. A. Ballou. 2013. Technical note: Determination of preanalysis storage temperature and time allowances for ex vivo innate immune responses. J. Dairy Sci. 96:384–389. doi:10.3168/jds.2012-5750

Silva, P. R. B., K. S. Machado, D. L. Da Silva, J. G. N. Moraes, D. H. Keisler, and R. C. Chebel. 2015. Effects of recombinant bovine somatotropin during the periparturient period on innate and adaptive immune responses, systemic inflammation, and metabolism of dairy cows. J. Dairy Sci. 98:4449–4464. doi:10.3168/jds.2014-8959

Silva, T. H., I. Guimaraes, P. R. Menta, L. Fernandes, D. Paiva, T. L. Ribeiro, M. L. Celestino, A. S. Netto, M. A. Ballou, and V. S. Machado. 2022. Effect of injectable trace mineral supplementation on peripheral polymorphonuclear leukocyte function, antioxidant enzymes, health, and performance in dairy cows in semi-arid conditions. J. Dairy Sci. 105:1649–1660. doi:10.3168/jds.2021-20624

Stilwell, G., and R. C. Carvalho. 2011. Clinical outcome of calves with failure of passive transfer as diagnosed by a commercially available IgG quick test kit. Can Vet. J. 52:524–526. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3078008/.

Tizard, I. 2018. Veterinary Immunology, 10th ed. St. Louis, MO: Elsevier.

Urie, N. J., J. E. Lombard, C. B. Shivley, C. A. Kopral, A. E. Adams, T. J. Earleywine, J. D. Olson, and F. B. Garry. 2018. Preweaned heifer management on US dairy operations: Part V. Factors associated with morbidity and mortality in preweaned dairy heifer calves. J. Dairy Sci. 101:9229–9244. doi:10.3168/jds.2017-14019

Wang, Y., W. Guo, H. Xu, K. Tang, L. Zan, and W. Yang. 2019. Melatonin suppresses milk fat synthesis by inhibiting the mTOR signaling pathway via the MT 1 receptor in bovine mammary epithelial cells. J. Pineal Res. 67:e12593. doi:10.1111/jpi.12593

Xu, L., W. Zhang, M. Kwak, L. Zhang, P. C. Lee, and J. O. Jin. 2019. Protective effect of melatonin against polymicrobial sepsis is mediated by the anti-bacterial effect of neutrophils. Front. Immunol. 10:1371. doi:10.3389/fimmu.2019.01371

Yost, C. C., M. J. Cody, E. S. Harris, N. L. Thornton, A. M. McInturff, M. L. Martinez, N. B. Chandler, C. K. Rodesch, K. H. Albertine, C. A. Petti, et al. 2009. Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates. Blood 113:6419–6427. doi:10.1182/blood-2008-07-171629

Yu, Q., S. C. Miller, and D. G. Osmond. 2000. Melatonin inhibits apoptosis during early B-cell development in mouse bone marrow. J. Pineal Res. 29:86–93. doi:10.1034/j.1600-079x.2000.290204.x