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Effects of *Morinda citrifolia* (Noni) on CD4$^+$ and CD8$^+$ T-Cell Activation in Neonatal Calves

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ABSTRACT

Developmental immaturity of the immune system renders neonatal calves vulnerable to infectious causes of morbidity and mortality. Juice made from the Morinda citrifolia fruit (noni) reportedly has immune enhancing, antibacterial, and antiinflammatory effects. The objective of this study was to evaluate the potential immune-modulating effects of feeding noni puree to neonatal calves on T-cell activation on CD4$^+$, CD8$^+$, and $\gamma\delta$ T cells. Sixteen newborn Holstein bull calves were acquired in pairs from local dairies and confirmed to have adequate passive transfer at 24 h of age. The calves were divided into 2 groups: group 1 ($n = 8$) was composed of control calves, and group 2 ($n = 8$) received 30 mL of noni puree twice daily in milk replacer. Blood samples were collected from each calf on d 0, 3, 7, and 14. Mitogen-induced activation of CD4$^+$, CD8$^+$, and $\gamma\delta$ T-cell receptor-positive T cells was evaluated by measurement of the up-regulation of the interleukin 2 receptor, CD25, using 2-color flow cytometry. Concanavalin A and phytohemagglutinin were used as global mitogens. Noni puree-fed calves had an increase in CD25 expression on concanavalin A-stimulated CD4$^+$ ($P = 0.03$ for parametric and nonparametric analysis) and CD8$^+$ T cells ($P = 0.04$ for parametric analysis) on d 3 of the study, or approximately 4 to 5 d postpartum. There was also an effect over time for CD8$^+$ T cells ($P = 0.03$ for nonparametric analysis). Further studies are warranted to determine the cellular mechanisms responsible for these findings and whether noni supplementation in dairy calves translates to improved health and well-being.

Key words: *Morinda citrifolia*, neonatal calf, noni, immunomodulation, T cell

INTRODUCTION

The developmental immaturity of the immune system of the neonatal calf presents a predisposing factor toward increased morbidity and mortality. The naïve immune system of a calf is constantly challenged by a variety of viral and bacterial pathogens found in its environment, and as a result, approximately 8.7% of dairy heifers born alive die before weaning (USDA, 2002).

The *Morinda citrifolia* fruit (noni) is a natural product that has a broad range of immune-enhancing effects, including antibacterial, antiinflammatory, analgesic, antioxidant, and antitumor effects (Wang et al., 2002; Furusawa et al., 2003). Noni has been shown to induce the release of several immune mediators, many of which have beneficial stimulatory effects and aid in the maturation of the neonatal immune system (Hirazumi and Furusawa, 1999).

Previously, we examined the effects of feeding calves noni puree for the first 2 wk of life on bacterial killing via an ex vivo whole-blood bactericidal assay (Schäfer et al., 2008). That study demonstrated that noni-supplemented calves had more *Escherichia coli* killing power at d 14 compared with control calves, and that enhanced bactericidal activity in noni-supplemented calves increased over time. We postulated that these results may have been due to noni positively affecting pathogen-associated molecule pattern recognition via Toll-like receptor 4, resulting in an increase in bacterial engulfment, phagocytosis, or both, or a cytokine-mediated effect via enhanced T-helper cell recognition of antigens expressed on the MHC II. Because both of these mechanisms result in the activation of T cells (whether directly or as a downstream

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effect), we decided to examine whether noni supplementation would also have an effect on T-cell activation in neonatal calves.

In the current study, we evaluated the effects of noni supplementation in newborn calves during the first 2 wk of life on T-cell activation in response to the potent mitogens concanavalin A (**ConA**) and phytohemagglutinin (**PHA**) by 2-color flow cytometry. The $CD4^+$, $CD8^+$, and γδ T-cell receptor-positive ($\mathbf{TCR}^+$) T cells were deemed activated if they expressed CD25, the interleukin (**IL**)-2 receptor, which plays a crucial role in lymphocyte proliferation and differentiation (Waters et al., 2003).

MATERIALS AND METHODS

Reagents

Before assay, acid citrate dextrose-A was prepared with 2.2 g sodium citrate (dehydrate), 0.8 g citric acid (monohydrate), 2.5 g dextrose, and 100 mL H_2O. A cell-lysing solution of pH 7.2 was prepared by dissolving 1.5 g (10.6 m*M*) Na_2HPO_4 and 0.32 g (2.7 m*M*) NaH_2PO_4 in 1 L H_2O. A restoring solution of pH 7.2 was similarly prepared by dissolving 1.5 g (10.6 m*M*) Na_2HPO_4, 0.32 g (2.7 m*M*) NaH_2PO_4, and 27 g (462.0 m*M*) NaCl in 1 L H_2O.

Animals

Animals for this project were obtained from 6 local dairies. The study and procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison. Within 12 h of birth, 16 newborn Holstein bull calves that had already received 4.0 L of pooled colostrum arrived in pairs at the veterinary medical teaching hospital, where they were housed in individual pens without nose-to-nose contact. The animals were assigned to control or treatment groups in the order in which they were removed from the calf trailer by hospital personnel, who had no further involvement with the study.

Upon arrival, every animal received a physical examination, followed by daily examinations for the duration of the study. At each examination, the following parameters were measured: temperature, ease of cough induction, fecal consistency, and presence and severity of ocular or otic abnormalities. A calf health score was assigned to each animal using the scoring system described previously in Schäfer et al. (2008). Veterinarians blinded to treatment groups oversaw calf health evaluations. Any calf receiving a total health score of ≥5 for 3 consecutive days was removed from the study and treated appropriately. Adequate passive transfer (IgG >1,000 mg/dL) was confirmed for all calves in the study with the IgG Midland Quick Test Kit (Midland Bioproducts Corp., Boone, IA) at approximately 24 h of age.

Calf pairs consisted of 1 noni puree-fed calf and 1 control calf. Calves were bottle-fed 2 L of nonmedicated milk replacer (Calf Glo, Vita Plus Corp., Madison, WI) reconstituted according to the label of the manufacturer twice daily for the first 7 d and 2.5 L twice daily from d 8 to 14. Noni puree-fed calves received 30 mL noni puree twice a day in milk replacer. Calves had access to 125 g calf starter and 4 L fresh water per day. Of the 8 calf pairs, only 3 had differing health scores between calves on d 0: in 2 pairs the score was 1 unit greater for the control calf, and in 1 pair it was 1 unit greater for the noni puree-fed calf. No calves were removed from the study because of health reasons.

Peripheral Blood Mononuclear Cell Isolation

The acid citrate dextrose-A anticoagulated blood (25 mL) was collected in Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) from the jugular vein from each calf on d 0, 3, 7, and 14. Day-0 samples were obtained from each calf between 36 and 48 h of age and before the first feeding of puree. All other samples were obtained before the respective feeding.

The blood was diluted 1:1 with PBS, and the buffy coat was harvested after centrifugation at 920 × *g* for 30 min at 25°C, diluted with plasma, layered over 1.083 Ficoll-Histopaque (Sigma, St. Louis, MO), and centrifuged at 1,380 × *g* for 30 min at 25°C. The peripheral blood mononuclear cell (**PBMC**) interphase layer was harvested and the remaining red blood cells were exposed to a lysing solution for 1 min, which was neutralized with a restoring solution (final ratio 1:1:1). The PBMC were washed twice, once with PBS and once with RPMI-1640 (Mediatech Inc., Herndon, VA) at 280 × *g* for 10 min, and were subsequently resuspended in RPMI-1640 with 20% fetal calf serum (**FCS**). Cells were stained with Trypan blue to confirm cell viability (>95%) and adjusted with RPMI-1640 with 20% FCS to 5×10^6 cells/mL.

Flow Cytometry

For flow cytometry analysis, 50 μL of PBMC (5×10^6 cells/mL) were cultured on a Costar flat-bottomed, tissue culture-treated, 96-well plate (Corning Inc., Corning, NY). Unstimulated control wells received 100 μL RPMI-1640 + 20% FCS. Stimulated sample wells received 100 μL ConA (final concentration of 6.7 μg/mL; Sigma) or 100 μL of the M form of PHA (Gibco, Invitrogen Corp., Carlsbad, CA) diluted 1:200 in RPMI-1640 + 20% FCS. Plates were incubated for 72 h at 37°C at 5% CO_2.

After incubation, the cells were transferred into 12 × 75 mm polystyrene round-bottomed tubes (Becton Dickinson) and washed with PBS at 520 × *g* for 7 min at 25°C. The cells were resuspended and stained for 2-color flow cytometry with the primary mouse anti-bovine antibodies: CD4 (IgM), CD8α (IgM), TCR1-N6 ($\gamma\delta$ TCR^+; IgM), and CD25 (IgG2a; 15 μg/mL; VMRD, Pullman, WA).

Stained cells were incubated for 20 min at 4°C and washed 3 times with PBS at 520 × *g* for 7 min at 25°C. The cells were resuspended again and stained with the secondary anitbodies: fluorescein isothiocyanate-conjugated

goat anti-mouse IgM and R-phycoerythrin-conjugated goat anti-mouse IgG2a (Jackson ImmunoResearch, West Grove, PA). The respective isotype antibodies were also tested to ensure there was no cross-reactivity. After a 20-min incubation at 4°C, cells were washed 3 times as before and resuspended in 500 μL PBS with 1% paraformaldehyde. Subsequently, a 2-color flow analysis was performed on a fluorescence-activated cell sorter flow cytometer (FACScan, Becton Dickinson) and the results were analyzed in FlowJo (version 8.1.1, Tree Star Inc., Ashland, OR).

Statistical Analysis

To normalize the data, calves were analyzed in pairs according to arrival date. Each pair came from the same farm, had been exposed to the same environment over the same time frame, and received the same pooled colostrum before arrival at the veterinary medical teaching hospital. This method resulted in n = 8 data pair sets for analysis at 4 time points.

For the analysis, the background was removed by subtracting the unstimulated control from the mitogen-stimulated value. Using the background-corrected data, we then computed the differences in proliferation for each calf pair (noni puree-fed − control) for both ConA and PHA, at each of 4 time points (0, 3, 7, and 14 d). This difference between noni-fed and control calves was used as the response in a repeated-measures ANOVA with a first-order autoregressive correlation structure to determine whether there was an effect of noni at each time or a trend in the effect of noni over the time period of the study. The *P*-values for mean differences between noni and the control at specific time points were corrected using the Bonferroni method; tests comparing the mean difference across time points were corrected using the Tukey method. Separate analyses were performed for ConA and PHA for CD4 and CD25, CD8 and CD25, and TCR1-N6 and CD25 staining combinations. All analyses were performed in PROC MIXED (SAS Institute Inc., Cary, NC). Significance was set at $P \leq 0.05$.

Residual analysis of the above models showed that the data were approximately normally distributed. However, because the sample sizes were quite low, assessing normality was difficult. To avoid an overly heavy reliance on the assumption of normality, nonparametric equivalents of all the normal-assuming tests were also performed. We used the Wilcoxon signed-rank test (PROC UNIVARIATE of SAS) to look at differences between noni and the control at each time point, and Friedman's test (PROC FREQ of SAS) to look at differences over time. Pair-wise tests following Friedman's test also used the Wilcoxon signed-rank test, and the *P*-values were Bonferroni corrected (Table 1).

Table 1. Percentage of expression of CD25 mean differences and parametric and nonparametric *P*-values[1]

			P-value	
Cell type	Time, d	Mean difference ± SEM	Parametric	Nonparametric
CD4	3	18.808 ± 5.042	0.03	0.03
CD8	3	14.105 ± 4.035	0.04	0.13
CD4	0 to 14	9.640 ± 4.046	0.80	0.29
CD8	0 to 14	8.252 ± 2.457	0.06	0.03

[1]Mean differences ± SEM, parametric and nonparametric *P*-values for the mean difference (noni − control) in CD25 percentage of expression on $CD4^+$ and $CD8^+$ T cells for both d 3 and over time (d 0 to 14) between calf pairs (n = 8) for concanavalin A-stimulated values with unstimulated controls (background) removed.

RESULTS AND DISCUSSION

Results showed an increase in percentage of expression of CD25 on $CD4^+$ T cells in response to ConA in noni-fed calves on d 3 of the study, or approximately 4 to 5 d postpartum ($P = 0.03$) when parametric analysis or nonparametric analysis ($P = 0.03$) was performed (Figure 1). An increase in the expression of CD25 on $CD8^+$ T cells for noni-fed calves was stimulated by the same mitogen at the same time point for parametric analysis ($P = 0.04$; Figure 1); however, significance was not present when the nonparametric analysis was performed. There was also an effect over time for $CD8^+$ T-cell activation in noni puree-fed calves when stimulated with ConA, with the highest mean difference at d 3, followed by a gradual decrease to d 14, when levels were approximately that of d 0 ($P = 0.03$) for the nonparametric analysis. There were no differences in $\gamma\delta$ TCR^+ cells or in the response to PHA within any T-cell subset (data not shown).

The leading management strategy to diminish calf morbidity and mortality is through the ingestion of high-quality colostrum to ensure adequate passive transfer of Ig that provide immediate protection against infection and disease until the immune system becomes fully functional (USDA, 2002; Sangild, 2003). In addition to Ig, colostrum includes growth factors, hormones, and leukocytes that also play a role in modulating neonatal immunity (Blum, 2003; Reber et al., 2005, 2006). It has been shown that these colostral cells and cytokines enter neonatal circulation and peak at 12 to 24 h after ingestion (Hagiwara et al., 2001; Liebler-Tenorio et al., 2002; Yamanaka et al., 2003a). This time point is approximately that of d 0 of this study and of gut closure, which occurs at approximately 1 to 2 d of age. Because the $CD8^+$ T-cell activation time effect in the noni-supplemented calves resembles the trafficking pattern and concentration of colostral cells and cytokines found in neonatal circulation after colostrum ingestion, we postulate that noni may

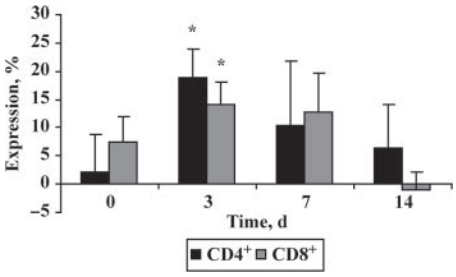

Figure 1. Percentage of expression of CD25 on $CD4^+$ and $CD8^+$ T cells. The figure depicts mean differences (noni − control) in CD25 percentage of expression on $CD4^+$ and $CD8^+$ T cells between calf pairs (n = 8) for concanavalin A-stimulated values with unstimulated controls (background) removed. The figure depicts the means + SEM. $^*P < 0.05$.

be affecting the absorbed maternal cells, cytokines, or both.

It is well established that the cytokines IL-2, tumor necrosis factor (**TNF**)-α, and interferon (**IFN**)-γ have immune-stimulatory effects through lymphocyte activation. Yamanaka et al. (2003b) suggested that the colostral cytokines IL-1β, TNF-α, and IFN-γ play a role in enhancing neonatal immunity through upregulation of IL-2 mRNA and the mature IL-2 receptor, CD25, perhaps in part because of an increased sensitivity of PBMC to colostral cytokines. Coincidentally, noni is capable of stimulating the release of IFN-γ, IL-1β, IL-10, IL-12 p70, TNF-α, and nitric oxide and suppressing IL-4 production from murine effector cells (Hirazumi and Furusawa, 1999). If noni also upregulates IL-1β, TNF-α, and IFN-γ in bovine colostrum, there may be a direct increase in natural cell-mediated immunity through the enhanced activation of $CD4^+$ and $CD8^+$ T cells.

Currently, it is not possible to distinguish whether the effect of this increase in CD25 on $CD4^+$ and $CD8^+$ T cells after noni puree feeding was mediated directly on T cells via endogenous cytokines or indirectly through other cellular immune components. It is also unknown whether the effect was on the neonatal cells, the maternal cells, or both. It will be important to perform further studies to elucidate the cellular components responsible for these findings. Additionally, a large clinical trial is warranted to determine whether these increased immune responses translate into increased health and well-being.

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