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Dynamics of immunity in Holstein calves during the neonatal period: evaluation of leukogram, cytokine gene expression, and T lymphocytes

Dinâmica da imunidade de bezerros holandeses no período neonatal: avaliação do leucograma, expressão gênica de citocinas e linfócitos T

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Abstract: The immune system of neonatal calves is immature and highly susceptible to diseases, which poses significant challenges to their survival. This study aimed to evaluate the immune response of calves during the first 30 days of life, focusing on leukogram analysis, T lymphocyte immunophenotyping (CD3+, CD4+, and CD8+) via flow cytometry, and cytokine gene expression of IL-10 and IL-12 through real-time PCR. The findings revealed that the calf immune system undergoes a postnatal adaptation process, as evidenced by variations in total and differential leukocyte counts, with a gradual increase in lymphocytes by day 30 and fluctuations in granulocytes and monocytes. The lowest percentages of T lymphocytes and the lowest CD4+ to CD8+ ratio were observed on the third day of life, followed by a gradual recovery. IL-10 expression was detected on days 1, 3, 10, and 25, whereas IL-12 expression was observed on days 1, 3, and 30. These cytokines indicate a dynamic balance between Th1 (pro-inflammatory) and Th2 (anti-inflammatory) responses, suggesting efficient immunological regulation to mitigate excessive inflammation and combat pathogens. Therefore, the calf immune system undergoes an adaptation and maturation phase, as evidenced by immune response modulation observed in leukocyte variations and cytokine expression.

Key-words: cattle; flow cytometry; immune development; RT-qPCR

Resumo: O sistema imunológico dos bezerros neonatos é imaturo e altamente suscetível a doenças, o que representa desafios para sua sobrevivência. Este estudo teve como objetivo avaliar a resposta imune de bezerros nos primeiros 30 dias de vida, com foco no leucograma, na imunofenotipagem dos linfócitos T (CD3+, CD4+ e CD8+) por citometria de fluxo e na expressão gênica das citocinas IL-10 e IL-12 por PCR em tempo real. Os resultados indicaram que o sistema imunológico dos bezerros passa por um processo de adaptação pós-natal, evidenciado por variações nos leucócitos totais e diferenciais, com aumento gradual de linfócitos até o 30º dia e flutuações em granulócitos e monócitos. As menores porcentagens de linfócitos T e a relação CD4+/CD8+ mais baixa ocorreram no terceiro dia de vida, com recuperação gradual. A expressão de IL-10 foi detectada nos dias 1, 3, 10 e 25, enquanto a IL-12 foi observada nos dias 1, 3 e 30. Essas citocinas indicam um equilíbrio dinâmico entre respostas Th1 (pro-inflamatórias) e Th2

(anti-inflamatórias), sugerindo uma regulação imunológica eficiente para controlar inflamações excessivas e combater patógenos. Conclui-se que o sistema imunológico do bezerro passa por uma fase de adaptação e maturação, com modulação da resposta imune observada nas variações nos leucócitos e na expressão das citocinas.

Palavras-chave: bovinos; citometria de fluxo; desenvolvimento imunológico; RT-qPCR

1. Introduction

The estimated number of calves born annually in Brazil is approximately 44.6 million [1]. According to Radostits et al. [2], the highest risk of death for calves occurs during the first two weeks of life. In Brazil, calf mortality rates can reach up to 25%, mainly due to respiratory tract diseases and diarrhea. The characteristics of calves' immune system during the neonatal period make them highly susceptible to diseases [3]. These illnesses negatively affect the profitability of the production chain, either due to animal death or the costs of care and treatment, while also diminishing neonatal welfare [2]. Research has primarily focused on the immunological behavior of calves during the neonatal period. However, most studies have analyzed immune activity in diseased calves or those responding to vaccine stimuli, while the immune activity of healthy calves remains underexplored [4;5;6;7;8;9;10].

Understanding the immunological behavior of neonatal calves is essential to reducing disease incidence, lowering costs, and improving animal welfare. This knowledge is crucial for implementing practices such as the proper administration of colostrum. Colostrum provides essential antibodies and promotes passive immunity, protecting calves from severe neonatal infections, including diarrhea and respiratory diseases [11,12]. These practices reduce the need for interventions, consequently lowering the costs of veterinary treatments and medications, while also preventing economic losses associated with high mortality rates [13]. Furthermore, reducing disease incidence improves calf welfare by minimizing stress and suffering, promoting healthy growth in a safer and more comfortable environment [14]. Therefore, effective immune management not only optimizes resources but also ensures better developmental conditions for these animals, aligning with welfare-oriented practices.

Thus, this study aimed to evaluate the dynamics of the immune response in neonatal calves during the first 30 days of life through analyses of total leukogram and its differentials, the ratio of circulating CD4+ to CD8+ T lymphocytes, and the production of Th1 and Th2 cytokine profiles.

2. Material and Methods

2.1 Ethics committee

This study was approved by the Ethics Committee on the Use of Animals of the Faculty of Veterinary Medicine and Animal Science (FMVZ), University of São Paulo (USP), São Paulo, SP, Brazil, under CEUA No. 2372210114 dated January 22, 2014.

2.2 Animals

Twenty healthy male Holstein Friesian calves, born from eutocic deliveries, were included in this study. The animals were housed in the Animal Facility of the Clinic for Cattle and Small Ruminants (CBPR) at FMVZ/USP, São Paulo, from the first day post-birth and remained there until 30 days of age.

2.3 Feeding

The calves were fed colostrum from a high-quality colostrum bank, evaluated with a colostrometer (lactodensimeter) specifically for the experiment. Colostrum was provided at a volume equivalent to 10% of body weight, divided into two feedings via bottle: the first within one hour of life and the second within the first 12 hours. Colostrum feeding continued for the first three days of life to ensure adequate passive immunity transfer. Subsequently, the calves were fed milk replacer until 30 days of age at a volume equivalent to 10% of body weight, divided into two daily feedings via bottle. Additionally, hay, commercial feed, and water were offered ad libitum. Animal health during the experiment was monitored via physical examinations and complete blood counts.

2.4 Sample collection

Blood samples were collected from each animal at 1 and 3 days post-birth (during colostrum intake) and then every 5 days from 5 to 30 days post-birth. This schedule was designed to monitor potential changes in the evaluated variables during the first month of life. Samples were collected via jugular venipuncture using a vacuum system with heparinized, silicone-coated tubes. These samples were labeled and transported under refrigeration to the laboratory of the Department of Clinical Medicine at FMVZ/USP.

2.4 Evaluation

Total and differential leukocyte counts were assessed using an electronic particle counter (Mindray® BC-2800 Vet). The ratio of circulating CD4+ to CD8+ T lymphocytes was measured using flow cytometry. Fifty thousand leukocytes from each sample were analyzed with a flow cytometer (FACSCalibur®; Becton Dickinson Immunocytometry Systems™, San Diego, CA). The obtained data were analyzed with the FlowJo® software (Tree Star™, Inc., Ashland, OR).

mRNA expression of IL-12 (indicative of a Th1 response) and IL-10 (indicative of a Th2 response) in circulating leukocytes was assessed using quantitative real-time polymerase chain reaction (qPCR) following the manufacturer's protocols (TaqMan® MGB probes, FAM™ dye-labeled, Applied Biosystems®, Foster City, CA). Relative gene expression was analyzed using the method described by Pfaffl (15). Values were calculated based on the ratio of the threshold cycle (CT) of each target gene to that of the reference gene (Beta-actin) and corrected for reaction efficiency.

2.5 Statistics

Descriptive statistics were applied to evaluate each variable in Holstein calves during the first 30 days post-birth (1, 3, 5, 10, 15, 20, 25, and 30 days of age). Normally distributed data were analyzed using repeated-measures models (16) to compare means over time for each variable, with Tukey's post-hoc test used to adjust *P*-values resulting for multiple comparisons. Medians of non-normally distributed data were compared using the Friedman test, with Dunn's post-hoc test applied to adjust *P*-values.

3. Results

The total leukocyte, granulocyte, lymphocyte, and monocyte counts (Table 1) varied across the studied time points. The total leukocyte count decreased sharply on day 15 post-birth (6.70 × 10^3 μL^{-1}), with the highest count observed on day 10 (11.50 × 10^3 μL^{-1}). Granulocyte counts were higher on day 1 (8.50 × 10^3 μL^{-1}) and day 10 (8.05 × 10^3 μL^{-1}) and lowest on day 15 post-birth (3.55 × 10^3 μL^{-1}). Monocyte counts also varied, with the lowest observed on day 3 post-birth (0.65 × 10^3 μL^{-1}) and the highest on day 30 (1.10 × 10^3 μL^{-1}). Lymphocyte counts increased significantly with age, reaching peak values on day 30 post-birth (3.0 × 10^3 μL^{-1}).

Table 1. Median values (with interquartile range: Q1–Q3) of total and differential leukocyte counts in leukograms of 20 Holstein calves during the first month of life.

Variable	Leukocytes (10^3 μL^{-1})			Lymphocytes (10^3 μL^{-1})			Monocytes (10^3 μL^{-1})			Granulocytes (10^3 μL^{-1})		
	Q1	Med	Q3	Q1	Med	Q3	Q1	Med	Q3	Q1	Med	Q3
Days post-birth												
1	7.55	10.95^{b}	14.65	1.05	1.45^{a}	1.90	0.60	0.80^{abc}	0.95	5.70	8.50^{b}	12.30
3	6.35	7.80^{a}	8.95	1.20	1.50^{a}	1.85	0.60	0.65^{a}	0.70	4.60	5.60^{ac}	6.90
5	6.60	7.15^{a}	8.6	1.55	2.00^{ab}	2.45	0.60	0.80^{abc}	1.00	3.60	4.30^{a}	5.35
10	7.95	11.50^{b}	14.75	1.80	2.00^{ab}	2.55	0.80	1.00^{c}	1.30	5.25	8.05^{a}	11.90
15	5.55	6.70^{a}	11.05	1.75	2.55^{bc}	3.20	0.60	0.80^{abc}	1.15	2.55	3.55^{abc}	6.60
20	7.40	9.15^{ab}	11.15	2.00	2.45^{bc}	2.80	0.80	1.00^{bc}	1.15	3.85	5.5^{ac}	7.20
25	7.25	8.45^{ab}	10.55	2.60	2.90^{c}	3.75	0.65	0.90^{abc}	1.10	3.50	4.60^{bc}	6.10
30	8.70	10.75^{b}	13.2	2.75	3.00^{c}	3.25	0.80	1.10^{c}	1.60	5.00	7.05^{c}	9.60
Overall	6.95	8.70	11.6	1.60	2.30	2.90	0.70	0.85	1.10	3.90	5.55	8.30
P-value		< 0.001			< 0.001			< 0.001			< 0.001	

Different lowercase letters indicate statistically significant differences between medians across ages.

The quantification of CD3+ lymphocytes, their subpopulations (CD3+ CD4+ and CD3+ CD8+), and the CD4+ to CD8+ lymphocyte ratio (CD4+/CD8+) are presented in Table 2. Significant differences were observed across evaluation days for CD3+, CD4+, and CD8+ lymphocyte quantifications ($P = 0.014$; $P < 0.001$; and $P < 0.005$, respectively). CD4+/CD8+ also varied significantly over the evaluation days ($P < 0.001$). The lowest percentages of CD3+, CD4+, and CD8+ lymphocytes were recorded on day 3 post-birth.

Furthermore, the highest percentage of CD3+ lymphocytes was observed on day 20 post-birth, whereas the highest percentages of CD3+ CD8+ lymphocytes were observed on days 5, 15, and 25 post-birth. CD3+ CD4+ lymphocytes reached their highest percentages on

days 25 and 30 post-birth. The lowest CD4+/CD8+ was observed on days 3 and 5 post-birth, while the highest ratio was recorded on day 30.

Tabela 2. Median values (with interquartile range: Q1–Q3) of circulating T lymphocyte (CD3+) subpopulation quantifications, obtained from 20 Holstein calves during the first month of life.

Variable	CD3+ (%)			CD3+ CD4+ (%)			CD3+ CD8+ (%)			CD4+ to CD8+ ratio		
	Q1	Med	Q3	Q1	Med	Q3	Q1	Med	Q3	Q1	Med	Q3
Days post-birth												
1	39.48	48.00^{ab}	61.41	14.68	21.28^{ab}	40.13	13.56	16.80^{ab}	19.07	0.88	1.32^{a}	1.98
3	39.37	45.94^{a}	57.39	4.82	12.06^{a}	20.43	10.28	13.82^{a}	18.77	0.59	0.89^{a}	1.24
5	49.68	54.09^{ab}	59.95	14.35	21.19a^{bc}	26.17	14.63	18.43^{b}	22.32	0.79	1.03^{ab}	1.71
10	32.96	55.71^{ab}	63.41	11.72	16.02^{ab}	25.91	13.48	15.83^{ab}	19.44	0.68	1.04^{a}	2.03
15	43.64	50.63^{ab}	61.60	12.04	21.17^{bc}	31.67	16.32	18.40^{ab}	20.23	0.74	1.22^{a}	1.43
20	52.61	66.35^{b}	72.43	16.99	22.32^{abc}	30.70	16.14	16.79^{b}	20.50	1.01	1.38^{ab}	1.57
25	52.79	59.15^{ab}	65.29	16.04	25.30^{bc}	38.12	13.59	18.46^{ab}	20.25	0.89	1.37^{ab}	1.98
30	51.98	52.96^{ab}	57.35	28.63	34.53^{c}	43.03	12.92	14.79^{ab}	16.84	1.67	2.71^{b}	3.06
Overall	43.18	53.90	62.73	12.56	21.28	30.66	13.81	16.73	19.57	0.84	1.23	1.85
P-Value		0.014			< 0.001			< 0.005			< 0.001	

Different lowercase letters indicate statistically differences between medians across ages, with P-values provided in the bottom row.

Cytokine gene expression was assessed using the model described by Pfaffl (15). Values were calculated based on the ratio of the threshold cycle (CT) of each target gene to that of the reference gene, corrected for reaction efficiency. Only one experimental group was analyzed, thus, day 1 post-birth was considered as the control, and values from subsequent time points were considered as experimental samples. Results were expressed as fold change. No significant differences in the gene expression of cytokines IL-10 and IL-12 in leukocytes were observed across the evaluation days (Table 3). Gene expression was undetectable at certain time points using the applied technique. IL-10 expression was detected on days 1 (control), 3, 10, and 25 post-birth, while IL-12 expression was detected on days 1 (control), 3, and 30 post-birth.

Table 3. Medians (with interquartile range: Q1–Q3) for the gene expression of cytokines IL-10 and IL-12 in leukocytes from 20 Holstein calves during the first month of life.

Variable	IL-10			IL-12		
	Q1	Med	Q3	Q1	Med	Q3
Days post- birth						
1		(1.00) Control			(1.00) Control	
3	0.726	1.673	3.955	0.776	1.221	1.631
5		—			—	
10	0.574	1.706	2.494		—	
15		—			—	
20					—	
25	1.839	3.597	3.927		—	
30		—		1.0435	1.939	1.947
P-Value		0.098			0.833	

The lowest lymphocyte counts in leukograms was observed on day 3 post-birth, with T lymphocyte subpopulations exhibiting decreased percentages of CD3+, CD4+, and CD8+ lymphocytes, as well as reduction in CD4+/CD8+. This was accompanied by the detection of interleukin 10 and 12 expression on this day.

An increase in monocytes and a decrease in granulocytes were observed on day 10 post-birth. Furthermore, there was an increased percentage of CD8+ lymphocytes, a decreased CD4+/CD8, and IL-10 expression on this day. On day 25 post-birth, IL-10 expression was detected, and lymphocyte counts in leukograms increased. A greater variation in CD8+ lymphocyte subpopulations was observed. IL-12 expression was detected at 30 days post-birth, accompanied by an increase in all leukocyte subpopulations. The percentages of CD4+ lymphocytes and CD4+/CD8+ also showed increased on this evaluation.

4. Discussion

The reduced levels of CD3+, CD3+ CD4+, and CD3+ CD8+ lymphocytes, along with a lower CD4/CD8 at 3 days post-birth, may be attributed to the abrupt exposure to the extrauterine environment, which presents a significant challenge to the neonate's immune system. This phenomenon is typically accompanied by an initial increase in neutrophils and decrease in lymphocyte levels, as observed in the leukograms of this study, indicating an acute inflammatory response and an adaptation period to the environment [17,18]. The gradual recovery in CD3+, CD3+ CD4+, and CD3+ CD8+ lymphocytes day 5 post-birth suggests ongoing immune system maturation in response to continued exposure to environmental antigens and microorganisms [19].

Considering the variations in leukograms, a trend toward the reversal of granulocyte-to-lymphocyte ratio at 30 days post-birth was observed, which is expected for animals at this age. Fluctuations in the leukogram during the first month of life are common and are associated with various factors, such as exogenous glucocorticoids from labor, maternal health, colostrum intake, and management and environmental factors [20].

The detection of IL-12 gene expression suggests activation of a Th1 response, likely due to exposure to microbial antigens after birth. This is supported by the leukogram results, which showed higher total leukocyte counts on days 1 and 30 post-birth, likely due to the large number of circulating granulocytes.

This is consistent with results from other studies that reported increased Th1 activity during similar neonatal developmental phases under bacterial infections [21]. IL-12 is crucial for the polarization of T cells toward a Th1 profile, which is essential for defense against early infectious pathogens [22]. Therefore, although not fully functional, the neonate's immune system is capable of responding when challenged by pathogens.

Furthermore, the detection of IL-10 expression on day 3 post-birth, followed by a decrease on day 5 post-birth, demonstrates a regulatory response aimed at balancing the activation of Th1 cells. A recent study reported that colostrum intake helps modulate the immune response in calves by inducing of IL-10 production, as a dysregulated production of pro-inflammatory cytokines in the intestine triggers local inflammatory processes, leading to systemic inflammation and tissue damage [23,24]. An increase in IL-10 was observed on day 10 post-birth, accompanied by an increase in total leukocyte counts. The anti-inflammatory function of this cytokine decreases the activity of leukocyte cells to prevent an excessive inflammatory effect [25].

The subsequent gradual increase in IL-10 expression may indicate the development of a balanced Th1/Th2 response as the immune system of the calves matures and adapts to the environment [26]. However, some studies have suggested that this shift toward a Th2 response can be attributed to immunomodulatory effects on the full-term calf. These effects include placental hormones, such as prostaglandin and progesterone, as well as cortisol from both the mother and the calf. The cumulative effect of these hormones causes the immune response to shift toward a Th2 profile, suppressing Th1 and stimulating the production of antibodies, particularly IgM [27].

The interaction between Th1 and Th2 cytokine responses during the first month of life appears to be crucial for the balanced and effective development of the immune system in calves [27,28]. The observed cytokine expression patterns suggest that, although an early Th1 response is essential for initial defense, subsequent modulation by Th2 cytokines is necessary to prevent excessive inflammation and promote an effective immune response, with antibody production as the calf's immune system develops and adjusts to the external environment [27,29].

Management strategies and therapeutic interventions can be more effective with a better understanding of this immune balance. For example, colostrum management can be optimized to improve immunoglobulin transfer and positively influence Th1 and Th2 responses [21,22, 27]. Additionally, nutritional interventions, such as supplementation with fatty acids or probiotics, have been shown to modulate immune responses in a beneficial way [30]. Regarding therapies, the targeted use of immunomodulators can help correct specific

imbalances, promoting a more effective response against pathogens while preventing damage from excessive inflammation [27, 31,32].

5. Conclusion

The immune system of Holstein calves undergoes an adaptation and maturation phase after birth. Although not fully functional at birth, it exhibits modulation of the immune response, as demonstrated by variations in leukocyte counts and their differentials, T lymphocyte percentages, and cytokine expression. The expression of the cytokines IL-12 and IL-10 suggest effective regulation between Th1 and Th2 responses, preventing an excessive inflammatory effect.

Conflict of interest statement

The authors declare no conflicts of interest.

Data availability statement

The data will be made available upon request to the corresponding author.

Author contributions

Conceptualization: C.L. Shecaira, F.J. Benesi, M.R. Azedo; Data Curation: C.L. Shecaira, M.R. Azedo; Formal Analysis: C.L. Shecaira, C.H. Seino; Funding Acquisition: C.L. Shecaira, F.J. Benesi; Project Administration:F.J. Benesi, M.R. Azedo; Supervision: F.J. Benesi, M.R. Azedo, P.E. Brandão, S. T. A. Miyagi; Investigation: C.L. Shecaira, M.R. Azedo, C.H. Seino, J.A Bombardelli, G.A. Reis; Validation: P.E. Brandão, S. T. A. Miyagi; Writing (Original Draft): C.L. Shecaira; Writing (Review and Editing): C.L. Shecaira

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