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SHORT COMMUNICATION

Effect of immune modulators and lactation number on in vitro proliferation of lymphocytes from nonpregnant dairy heifers and cows

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Abstract: The proliferation of blood lymphocytes from nonpregnant, nonlactating heifers was comparable with that of nonpregnant cows in their first lactation. Both low and high levels of β-hydroxybutyrate and low levels of prolactin, but not isoproterenol, reduced the proliferative response of dairy heifers and cows in their first or second lactation.

Key words: immune response, β-hydroxybutyrate, adrenaline, prolactin, cattle.

Résumé : La prolifération des lymphocytes sanguins des génisses non gestantes et non allaitantes était comparable à celle des vaches non gestantes à leur première lactation. De faibles et de fortes concentrations de β-hydroxybutyrate ainsi que les faibles concentrations de prolactine, mais pas l'isoprotérénol, ont réduit la réponse proliférative des génisses laitières et les vaches à leurs premières ou deuxièmes lactations. [Traduit par la Rédaction]

Mots-clés : réponse immunitaire, β-hydroxybutyrate, adrénaline, prolactine, bovins.

Lactation is associated with a higher incidence of disease due to profound metabolic and hormonal changes, which can directly or indirectly impair immune function in a multifactorial way. In dairy cows, negative modulators of immunity, such as β-hydroxybutyrate (BHB) and adrenaline, are increased during lactation, whereas only a few positive immune modulators, such as prolactin, are elevated. Therefore, nonlactating heifers might have a higher or at least a similar immune response as lactating primiparous cows. During lactation, peripheral blood mononuclear cells (PBMCs) from multiparous cows showed a lower proliferative response to mitogen stimulation than that of primiparous cows (Kashiwazaki et al. 1985). The reduced capacity of the immune system in multiparous cows to respond to pathogens might be related to energetic load that accumulates during closely spaced pregnancies and lactations with increasing milk yield (Mehrzad and Zhao 2008). This energetic load was demonstrated by differences between primiparous and multiparous dairy cows in a number of metabolic parameters, milk yield, and body condition, as well as their inter-relationships (Wathes et al. 2007). Accordingly, increased concentrations of BHB or isoproterenol (adrenaline-like substance) diminished the responsiveness of immune cells in a dose-dependent manner (Sempere et al. 2004; Schulz et al. 2015). In contrast, increased plasma concentrations of prolactin facilitated the in vitro proliferation of murine and human lymphocytes (Hartmann et al. 1989). Therefore, the present study examined the effect of immune modulators on immune responsiveness in heifers and cows. The effect of parturition or pregnancy on immune function was excluded by studying only cows in late lactation and nonpregnant animals. We

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hypothesized that lactation number (0, 1, and 2) and the concentration of BHB, isoproterenol, and prolactin (none, low, and high) affect in vitro proliferation of PBMCs.

The experiment was conducted in compliance with the German legislation on the use of experimental animals (LALLF M-V/TSD/7221.3-1.1-038/12). Four 20-mo-old, nonlactating Holstein heifers and eight Holstein dairy cows in their first ($n = 5$) or second ($n = 3$) lactation were investigated during winter. Cows in their first and second lactation were 180 ± 7 and 282 ± 90 d in milk, respectively. All animals were nonpregnant and clinically healthy. They were housed in free stall barns and were given ad libitum access to water and feed. Cows were fed twice daily a total mixed ration of maize and grass silage, dairy concentrate, maize, beet pulp, rapeseed and soybean meal, hay (1 kg d^{-1}), minerals, and lime. Heifers received grass silage with 1 kg d^{-1} hay and minerals (100 g d^{-1}). Cows were milked at 0530 and 1730, and milk yield was recorded.

Reagents were purchased in Germany from Biochrom (Berlin), Greiner-Bio One (Frickenhausen), PAN Biotech (Aidenbach), and Sigma–Aldrich (Taufkirchen). Peripheral blood mononuclear cells were prepared by density gradient centrifugation. Ice-cold sodium heparinized blood (vena jugularis) was mixed at 1:3 with 0.83% sodium chloride and arranged in a layer over a diatrizoate-Ficoll gradient buffered with 5 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.5 mmol L^{-1} disodium ethylenediaminetetraacetic acid, pH 7.3. The floating PBMCs were washed, cleared from erythrocytes by hypotonic lysis, and resuspended in complete medium (RPMI-1640 medium without phenol red and supplemented with 10 mmol L^{-1} HEPES, 2 mmol L^{-1} glutamine, and 10% fetal calf serum). Peripheral blood mononuclear cells were seeded onto 96-well microplates in quadruplicate per treatment after adjusting the cell concentration to 1×10^6 cells mL^{-1} using a cell counter (Beckman Coulter).

Cell proliferation was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay as described by Tuchscherer et al. (1998). The mitogen phytohemagglutinin (4 μg mL^{-1}) and the modulators such as isoproterenol hydrochloride (R+/−; 70 and 130 ng L^{-1}), prolactin (from sheep pituitary; 20 and 300 ng mL^{-1}), and sodium DL-β-hydroxybutyrate (0.5 and 3.0 mmol L^{-1}) were used. The concentrations of the modulators were chosen according to the reported plasma levels of cows around calving. Optical density (OD) was measured with a plate reader (MR5000, Dynatech, Denkendorf, Germany) using test and reference wavelengths of 550 and 690 nm, respectively. The proliferation index (PI) was calculated as the ratio of the ODs obtained in the presence and absence of mitogen.

The PI data were approximately normally distributed (UNIVARIATE procedure) and were analyzed by repeated measures analysis of variance with the MIXED procedure (SAS software version 9.4, SAS Institute Inc., Cary, NC, USA). The model contained the fixed factors treatment (control, low or high isoproterenol, low or high prolactin, and low or high BHB), lactation (nonlactating heifers and cows in their first or second lactation), and the treatment × lactation interaction. Treatments of cells from the same animal were considered a repeated measure. Pairwise multiple comparisons among the least-square means were performed by the Tukey–Kramer test.

During the first lactation (late-lactating stage), the hormonal and metabolic changes had no adverse effects on the capacity of PBMCs to respond to a stimulus because the PI of PBMCs from heifers was similar to that of the first lactating cows (Fig. 1). The in vitro PI of PBMCs from heifers and cows in their first and second lactation did not differ significantly ($P > 0.05$, F test and Tukey–Kramer test). In pairwise comparisons without alpha adjustment, the PI of PBMCs from cows in their second lactation was lower ($P = 0.047$) than the PI of cows in their first lactation (1.37 ± 0.32 vs. 2.11 ± 0.56). The higher PI observed in PBMCs from cows in their first lactation than in their subsequent lactations is in agreement with previous reports on the proliferative response of PBMCs from Holstein dairy cows, which used different assays and mitogens (Table 1). A difference in the PBMC response has been observed between primi- and multiparous cows that are in early lactation and not pregnant (Kashiwazaki et al. 1985; Schulz et al. 2015), in mid-late lactation and pregnant (Mehrzad and Zhao 2008; Tienken et al. 2015), and in late lactation and not pregnant (present study). In the studies of Kashiwazaki et al. (1985) and Mehrzad and Zhao (2008), and in the present study, the PI of cows with two or more lactations was 30% lower than that of cows in their first lactation. Tienken et al. (2015) and Schulz et al. (2015) observed a reduction of only 10% in the PI of PBMCs. The relative differences in the proliferative response of the studies listed in Table 1 are likely to be related to the variation in assays and mitogens. The lack of interaction between modulator treatment and lactation number implies that the treatment caused a similar proliferative response in heifer and cow PBMCs.

Compared with the unsupplemented control, low and high levels of BHB and low levels of prolactin slightly reduced ($P < 0.05$) the in vitro proliferation of PBMC from dairy heifers and cows in their first and second lactation (Fig. 1). Unexpectedly, isoproterenol (a β-adrenergic receptor agonist) did not impair the responsiveness of PBMCs at any dosage. Higher doses might be required to suppress proliferation. Other studies with bovine PBMCs are not available, but Sempere et al. (2004) observed a dose-dependent effect of isoproterenol on rat blood lymphocytes with concentrations of 2.23 mg L^{-1} and greater, i.e., >7-fold higher doses than used in the present study.

Prolactin can facilitate immune function, but in the present study, the PI of PBMCs did not increase with

Fig. 1. Effect of treatment and lactation number on the in vitro proliferation index of peripheral blood mononuclear cells from nonpregnant heifers and cows. The statistical analysis included the treatment (control, 70 or 130 ng L^{-1} isoproterenol, 20 or 300 ng mL^{-1} prolactin, and 0.5 or 3.0 mmol L^{-1} β-hydroxybutyrate), lactation number (0, 1, and 2), and the interaction of these variables. Least-square means between treatments (thick solid lines) without a common letter differ at $P < 0.05$.

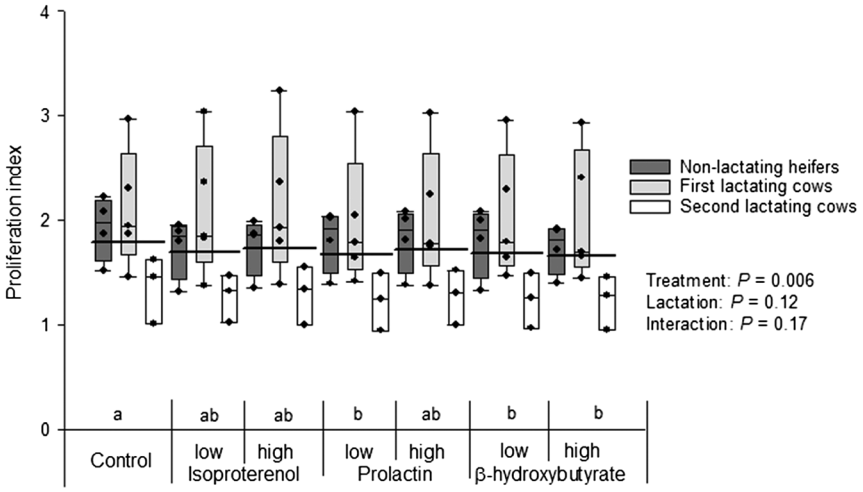

Table 1. In vitro proliferation index of peripheral blood mononuclear cells from Holstein cows with different lactation numbers.

Publication/lactation number	Proliferation index[a]	Assay, Mitogen	Animal number	Days in milk	Milk yield (kg d^{-1})
Kashiwazaki et al. 1985					
1	7.5	^{3}H-thymidine, PHA	4	7–14	NS
2–6	5.5		5		
Mehrzad and Zhao 2008					
1	4.3	CFDASE, ConA	13	225 ± 86	30 ± 2
4–6	2.9		13	196 ± 58	41 ± 2
Tienken et al. 2015					
1	5.4	Alamar blue, ConA	5	100	NS
≥2	4.8		7		
Schulz et al. 2015					
1	6.1	Alamar blue, ConA	8	7	26 ± 1
≥2	5.5		9		
Present study					
0	1.9	MTT, PHA	4	NA	NA
1	2.1		5	180 ± 7	29 ± 2
2	1.4		3	282 ± 90	29 ± 7

Note: PHA, phytohemagglutinin; CFDASE, carboxyfluorescein diacetate succinimidyl ester; ConA, concanavalin A; MTT, 3-[4,5-imethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; NA, not applicable; NS, not specified.

[a]Average ratio of the response in the presence and absence of the mitogen, or the response to mitogen alone (Kashiwazaki et al. 1985).

increasing prolactin levels. In contrast, low prolactin levels slightly reduced the immune response compared with the unsupplemented control. This is unlike in humans and mice, in which a lack of prolactin inhibited lymphocyte proliferation in vitro (Hartmann et al. 1989). Other articles on cow PBMCs are not available, but prolactin is generally mitogenic for lymphocytes due to its shared properties with hematopoietic growth factors. The nonmitogenic effect of prolactin in the present study might be attributed to a downregulation of prolactin receptors in PBMCs (Auchtung et al. 2003). Difficulties in demonstrating the mitogenic effect of prolactin on immune cells are not unusual (Lessard et al. 2005).

Unexpectedly, PBMCs from nonlactating heifers and cows in their first and second lactation did not differ in their response to BHB. The mechanisms by which BHB

impairs immune cell function are currently unknown. Our results suggest that the immunomodulatory activity of BHB is independent of lactation number. Similar to our findings, Targowski and Klucinski (1983) also reported an inhibition of in vitro PBMC proliferation with comparable low and high concentrations of BHB (0.8, 4.0 mmol L^{-1}) in mid-lactating cows. In other studies, only markedly higher levels of BHB impaired the proliferation of PBMCs from nonpregnant heifers (6.25, but not ≤1.25 mmol L^{-1}, Franklin et al. 1991) and from early-lactating cows (≥5.0, but not ≤2.5 mmol L^{-1}, Schulz et al. 2015). Peripheral blood mononuclear cells from early lactating cows might use lower levels of BHB as an alternative (or additional) energy source, whereas the same levels are immunosuppressive for PBMCs originating from mid- and late-lactation cows. For nonpregnant heifers, the results are divergent, and further studies are warranted.

Overall, the comparable proliferative response of PBMCs from nonlactating Holstein heifers and cows in their first lactation implies that the hormonal and metabolic state during the first lactation (late-lactating stage) has no adverse effect on the average proliferation potential. Despite the different endocrine backgrounds of PBMCs from heifers and dairy cows, we have shown that prolactin and BHB similarly modulate the cellular immune response to mitogenic stimulation, as shown by a slight decrease in the PBMC proliferative response.

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