

LOWER SERUM FREE AMINO ACIDS IN HOLSTEIN COWS WITH MILK FEVER IN FIELD CASE

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This study was conducted to investigate the relationship between serum free amino acids (FAA) and the onset of milk fever in dairy cows around the time of calving. The serum concentration of FAA was compared between multiparous cows with milk fever (MF group; n=7) and healthy multiparous cows (Control Group; N=16) kept on a Holstein dairy farm. Blood samples were obtained 3 to 4 weeks prepartum, on the day of calving, and 1 month post partum for analysis of serum amino acids and lipid parameters. The levels of all essential amino acids in the MF group were lower than those in the Control group. Significant differences between groups were observed in phenylalanine, valine, isoleucine, and leucine at 1 month post partum, and in tryptophan on the day of calving. Regarding the non essential amino acids, a significantly lower level of arginine was observed in the MF group compared to the Control group on the day of calving, as well as 1 month post partum. A significant positive correlation was detected between cholesterol and branched chain amino acids (BCAA) levels ($r=0.531$, $P<0.01$). These results showed that MF cows had lower levels in several FAA around the time of calving even before the onset of MF.

Keywords: calving, dairy cow, free amino acids, milk fever

INTRODUCTION

During late gestation, dry matter intake (DMI) of dairy cows is quite low whereas nutrient demand is extremely high [1]. Because nutritional requirements increase at the end of gestation to meet the demands of the fetus inside the uterus and growing mammary gland for the next lactation, pregnant cows need to mobilize energy from reserves [2]. However, serious reductions in DMI near parturition predispose animals to metabolic disorders such as displaced abomasum, milk fever, and ketosis. A decrease in nutritional levels, such as albumin, glucose, and cholesterol during the dry period was observed in the dairy herd with a high incidence of periparturient diseases [3,4].

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Thus, deficiency of nutrients around the time of calving may affect the onset of periparturient diseases.

Animals obtain essential amino acids (EAA) from feed and convert them to non essential amino acids (NEAA) that are used as energy sources or for protein synthesis inside the body. In dairy cows, maintaining a good balance of amino acids is important for achieving higher milk yields and good health [5].

A previous study reported higher serum total free amino acids in cows following higher DMI after calving [6]. On the other hand, lower levels of serum nutritive parameters such as cholesterol or beta hydroxybutyric acid in the preparturient period were often associated with negative effects on postparturient health conditions, underscoring the importance of nutritional improvement [7,8]. In particular, peripartum amino acids deficiency is known as a risk factor for metabolic diseases in the postpartum period [9,10]. A deficiency of protein energy is a form of malnutrition, leading to a shortage of amino acids in all cells in the body. The cows developed retained placenta after calving showed low circulating amino acids during the last 2 weeks of gestation [11]. The constituents of free amino acids in serum with the onset of milk fever in dairy cows around the time of calving have not been fully clarified. The objective of the present study was to investigate the changes in free amino acids and lipids in the cows which developed milk fever during the dry and early lactation periods in field conditions.

MATERIALS AND METHODS

Animals and protocol design

Twenty-three multiparous Holstein cows kept in one farm were used in this study. Seven cows had an onset of milk fever (MF group; mean parity 4.86 ± 0.95) and 16 cows remained clinically healthy throughout the experimental period (Control group; mean parity 3.00 ± 0.25), which showed a significant difference in parity ($P < 0.05$). Mean milk yield per day 1 month after calving was 42.59 ± 1.58 kg in the MF group and 47.98 ± 0.95 kg in the Control group, without significant difference between groups. The MF group showed clinical signs such as lethargy, no appetite, low temperature of the body surface, weak heartbeat, muscular weakness, unable to stand, and relaxation of the anus. The level of serum calcium was under 5.5 mg/dl in all cows of the MF group before treatment. The response to intravenous calcium infusion in the cows in the MF group was rewarding, and the cows with MF recovered after treatment with calcium containing infusion. No cows had a complication of other diseases, such as calving paralysis, fractures of bones or pelvis, hip luxation, coliform mastitis, hypovolemic shock, and none were treated for other diseases during the experimental period. There were no instances of death or culling.

All dry cows were kept in free stall housing. They were fed about 17 kg of total mixed ration (TMR), and had free access to hay and water. All cows in late gestation were moved from close up stalls to the maternity pen prior to the onset of labor, where they delivered. The lactating cows were kept in another free stall housing with free access to water. The feeding components of TMR during the dry and lactating periods are shown in Table 1.

Table 1. Composition of the diets fed to the cows during the dry and lactation period

Item	Diet(kg/day)	
	Dry period	Lactating period
Corn silage		24.9
First-cut grass silage		11.8
Second-cut grass silage	15.0	6.0
Dry ground corn		3.2
Soybeen meal		1.4
Bypass protein	0.5	0.6
Compound feed	1.0	7.0
Beet pulp		2.0
Vitamin-mineral mix ¹		0.5
Vitamin-mineral mix ²	0.2	

¹Vitamin-mineral mix contained: 0.18g sodium chloride, 0.19g calcium carbonate, 0.01g magnesium oxide, 0.9g vitamins;

²Vitamin-mineral mix contained: 0.19g calcium carbonate, 0.05g vitamins

Blood sampling and analyses

Blood samples were collected from the tail vein into plain vacutainer tubes at 3 to 4 weeks before calving (Dry), on the day of calving, and at 1 month after calving. Blood samples were immediately centrifuged at 3,000 rpm for 10 min for isolating serum. The serum was kept at -30 °C until analysis.

The concentration of FAA in the serum was analyzed using an amino acid automatic analyzer (JIC-5/V, Nippon Electron, Co., Ltd., Tokyo, Japan). Serum samples were used to measure the serum total cholesterol (TC), beta hydroxy butyric acid (BHB), and non esterified fatty acids (NEFA) by enzyme assay. Body condition Score (BCS) was evaluated according to the method of Ferguson et al. [12].

Ethics

In this study, the procedures used were in accordance with the principles and guideline for animal use set by the Animal Experiment and Care Committee of Obihiro University of Agriculture and Veterinary Medicine Obihiro, Japan (No.24-4).

Statistical analysis

Analyzes were performed using the software-Excel TOKEI version 2.02 (SSRI Co., Ltd. Tokyo, Japan). All data are expressed as mean $\pm$ standard errors. All data were not normally distributed, and statistical analysis for each parameter was performed using Mann Whitney U test in order to determine the difference between two groups at the same sampling time. The difference was considered significant at $P<0.05$. Correlations of time course changes between biochemical parameters and FAA were analyzed using Spearman's rank correlation test, and significance was determined at $P<0.01$ or $P<0.05$.

RESULTS AND DISCUSSION

In this study, most amino acids in the MF group were lower at all sampling points, and significant differences were found in TAA, EAA, BCAA, and AAA during the Dry period (Table 2).

Table 2. Serum free amino acids concentration ($\mu\text{mol/L}$) in the tested groups

		MF Group	Control Group	
TAA	Dry	3210.2 $\pm$ 470.2	6031.9 $\pm$ 1104.0	*
	after calving	3097.2 $\pm$ 554.8	5514.4 $\pm$ 809.9	*
	1 Month	4266.2 $\pm$ 842.5	6599.5 $\pm$ 667.1	
EAA	Dry	1281.0 $\pm$ 126.3	1898.3 $\pm$ 170.4	*
	after calving	989.6 $\pm$ 175.6	1429.1 $\pm$ 166.0	
	1 Month	1396.5 $\pm$ 239.6	2137.9 $\pm$ 206.6	*
NEAA	Dry	1603.4 $\pm$ 371.4	2416.0 $\pm$ 552.7	
	after calving	1694.0 $\pm$ 339.6	2291.9 $\pm$ 351.4	
	1 Month	2448.7 $\pm$ 634.4	2804.1 $\pm$ 371.0	
AAA	Dry	120.1 $\pm$ 12.0	165.5 $\pm$ 14.5	*
	after calving	106.0 $\pm$ 15.8	146.8 $\pm$ 17.1	
	1 Month	120.7 $\pm$ 14.8	169.4 $\pm$ 17.6	
BCAA	Dry	681.2 $\pm$ 70.2	987.2 $\pm$ 99.9	*
	after calving	489.5 $\pm$ 92.7	641.7 $\pm$ 77.8	
	1 Month	815.9 $\pm$ 159.1	1241.1 $\pm$ 138.1	

Data are expressed as mean $\pm$ SE.
Astarisk indicate significant difference between the two tested groups ($P < 0.05$).
TAA=Total amino acid, EAA=Essential amino acid, NEAA=Non-essential amino acid,
AAA=Aromatic amino acids, BCAA=Branched-chain amino acids.

It suggested that the main reason for decreased serum TAA was not NEAA but decreased EAA, since no significant difference in NEAA was found. Serum TAA of periparturient dairy cattle are influenced by EAA and NEAA as well as milk yield and

feed intake [13]. Additionally, the placenta and the fetus in pregnant dairy cows are the major users of EAA. EAA seems to be used for the synthesis of NEAA rather than energy supply [14].

Zhang et al. [15] described that DMI and milk production were lower in MF affected cows close to calving. In this study, markedly lower serum FAA levels in the MF Group were observed in the Dry period. Free amino acids deficiency is caused by a lack of amino acids in the body. Bell [16] reported in ruminants that 35-40% of the energy supply to the placenta depends on glucose and lactate, and 55% on amino acids during the last third of pregnancy. In particular, proteins in the prepartum diet have a critical role in fetal protein synthesis from amino acids and as a source of a substantial amount of energy. It suggests that protein and amino acid intake might be insufficient before calving in the MF group to meet increased demands of amino acids for growing fetuses in late pregnancy.

Table 3 shows the concentration of essential amino acids in two groups. All values of EAA in the MF Group were also lower than those in the Control group. Significant differences were detected in tryptophan in the dry period and at 1 month, as well as in phenylalanine, valine, isoleucine, and leucine during the dry period. A similar result was reported previously in the dams with a restricted protein diet [17]. BCAAs are considered to be the critical amino acids for anabolism and energy homeostasis of the skeletal muscle serving as signaling molecules [18]. Since BCAAs are one of the key nutrients for muscle repair and development by synthesizing muscle proteins from plasma amino acids, deficiency of these amino acids might reduce the muscle function in the cows in the MF group around the time of calving. But in comparison to non essential amino acids, concentrations of arginine, glycine, aspartic acid, glutamic acid, and glutamine in the MF group were lower than those in the Control group. Significant differences were found in arginine at calving and 1 month post partum (Table 4).

Although the lowest level of serum TAA was observed in both groups on the day of calving, previous report indicated that decreased FAA was associated with a normal decrease in voluntary dry matter intake (DMI) prior to the day of calving [13]. In the present study, significantly positive correlations of cholesterol to BCAA ($r=0.531$, $P<0.01$), as well as to valine, leucine, and isoleucine levels ($r=0.563$, $r=0.563$, and $r=0.403$, respectively, $p<0.01$) were detected. This change is in accordance with previous reports [19-21], and may be due to the decreased dietary intake around the time of calving. Since Zhou [5] observed higher serum cholesterol and total amino acid including valine and isoleucine in the cows following their higher DMI after calving, DMI was suggested to be one of the factors to affect the changes to these nutrients.

Arginine is a conditionally essential amino acid that plays key roles in nitrogen metabolism, ureagenesis, cellular multiplication, and energy metabolism [22]. This amino acid protects muscle protein metabolism during stress induced hypercatabolism. In this study, a markedly low level of arginine was observed in the MF group after calving. Since arginine is involved in multiple metabolic processes in the body, this amino

acid deficiency has the potential to depress many cellular and organ functions [23]. Previous studies have reported that injection of arginine via the jugular vein alleviated metabolic stress in Holstein cows during early lactation [24]. Thus, arginine deficiency could induce depressed function of muscles, mammary glands or other organs.

Table 3. Serum free essential amino acids concentration ($\mu\text{mol/L}$) in the tested groups

		MF Group	Control Group	
Methionine	Dry	27.8 $\pm$ 2.4	37.2 $\pm$ 3.7	
	after calving	30.5 $\pm$ 6.9	38.5 $\pm$ 6.0	
	1 Month	20.9 $\pm$ 3.4	31.0 $\pm$ 5.1	
Lysine	Dry	118.4 $\pm$ 13.5	163.1 $\pm$ 19.2	
	after calving	73.7 $\pm$ 10.7	114.9 $\pm$ 14.6	*
	1 Month	106.3 $\pm$ 17.9	154.3 $\pm$ 15.1	
Threonine	Dry	99.2 $\pm$ 9.7	123.9 $\pm$ 12.3	
	after calving	68.9 $\pm$ 13.0	99.5 $\pm$ 12.6	
	1 Month	94.7 $\pm$ 10.7	114.1 $\pm$ 9.2	
Histidine	Dry	76.6 $\pm$ 0.5	109.5 $\pm$ 14.1	
	after calving	73.3 $\pm$ 17.3	108.8 $\pm$ 15.7	
	1 Month	67.7 $\pm$ 7.3	81.7 $\pm$ 7.8	
Tryptophan	Dry	36.1 $\pm$ 6.7	63.3 $\pm$ 9.8	*
	after calving	22.4 $\pm$ 6.6	59.1 $\pm$ 17.8	
	1 Month	36.1 $\pm$ 7.9	125.6 $\pm$ 33.3	*
Phenylalanine	Dry	60.5 $\pm$ 6.6	87.5 $\pm$ 9.6	*
	after calving	62.1 $\pm$ 9.6	84.3 $\pm$ 10.5	
	1 Month	59.7 $\pm$ 6.5	87.7 $\pm$ 12.6	
Valine	Dry	334.0 $\pm$ 32.7	485.3 $\pm$ 45.3	*
	after calving	216.2 $\pm$ 42.3	281.2 $\pm$ 33.8	
	1 Month	384.7 $\pm$ 76.5	566.7 $\pm$ 56.0	
Isoleucine	Dry	151.4 $\pm$ 15.7	219.0 $\pm$ 24.2	*
	after calving	119.8 $\pm$ 27.9	150.3 $\pm$ 19.5	
	1 Month	220.5 $\pm$ 44.1	345.5 $\pm$ 40.9	
Leucine	Dry	195.7 $\pm$ 22.3	282.9 $\pm$ 31.1	*
	after calving	153.5 $\pm$ 26.6	210.2 $\pm$ 26.4	
	1 Month	210.7 $\pm$ 39.0	328.5 $\pm$ 43.5	

Data are expressed as mean $\pm$ SE

Astarisk indicate significant difference between the two tested groups groups ($P < 0.05$).

Table 4. Serum free non-essential amino acid concentration ($\mu\text{mol/L}$) in the tested groups

		MF Group	Control Group	
Arginine	Dry	181.3 $\pm$ 19.4	296.5 $\pm$ 39.5	
	after calving	169.3 $\pm$ 39.5	286.1 $\pm$ 38.9	*
	1 Month	195.1 $\pm$ 35.7	302.3 $\pm$ 36.9	*
Glycine	Dry	248.2 $\pm$ 38.0	303.6 $\pm$ 30.8	
	after calving	427.0 $\pm$ 68.0	664.6 $\pm$ 78.4	
	1 Month	522.4 $\pm$ 72.3	762.0 $\pm$ 73.9	
Alanine	Dry	234.4 $\pm$ 24.5	288.3 $\pm$ 23.1	
	after calving	243.8 $\pm$ 41.9	335.2 $\pm$ 40.1	
	1 Month	287.8 $\pm$ 33.6	313.4 $\pm$ 27.8	
Asparagine	Dry	56.3 $\pm$ 21.2	69.5 $\pm$ 16.3	
	after calving	37.9 $\pm$ 8.3	54.4 $\pm$ 8.9	
	1 Month	128.8 $\pm$ 52.6	100.1 $\pm$ 25.3	
Aspartic acid	Dry	14.7 $\pm$ 3.6	28.3 $\pm$ 5.4	
	after calving	12.8 $\pm$ 3.3	22.0 $\pm$ 3.8	
	1 Month	14.3 $\pm$ 2.4	17.7 $\pm$ 1.8	
Glutamic acid	Dry	119.0 $\pm$ 13.3	176.1 $\pm$ 25.9	
	after calving	75.4 $\pm$ 13.5	116.9 $\pm$ 15.6	
	1 Month	92.8 $\pm$ 11.5	130.7 $\pm$ 13.9	
Glutamine	Dry	256.7 $\pm$ 13.3	382.2 $\pm$ 41.0	
	after calving	305.1 $\pm$ 69.8	451.2 $\pm$ 55.6	
	1 Month	263.4 $\pm$ 27.2	294.2 $\pm$ 25.3	
Serine	Dry	72.9 $\pm$ 8.6	96.7 $\pm$ 9.1	
	after calving	97.6 $\pm$ 20.2	134.6 $\pm$ 14.4	
	1 Month	105.5 $\pm$ 11.3	112.0 $\pm$ 8.4	
Proline	Dry	473.2 $\pm$ 312.4	893.7 $\pm$ 493.1	
	after calving	420.1 $\pm$ 178.6	409.9 $\pm$ 203.2	
	1 Month	924.3 $\pm$ 513.3	942.7 $\pm$ 258.4	
Tyrosine	Dry	59.6 $\pm$ 6.1	78.0 $\pm$ 6.1	
	after calving	43.9 $\pm$ 7.4	62.5 $\pm$ 7.2	
	1 Month	60.9 $\pm$ 8.8	76.0 $\pm$ 6.1	

Data are expressed as mean $\pm$ SE Astarisk indicate significant difference between the two tested groups ($P < 0.05$).

In the MF group, tryptophan was also markedly lower at 1 month after calving compared to the Control group. Ninomiya et al. [2] observed declined weights of body and skeletal muscle in mice fed a tryptophan deficient diet. BCS in both groups decreased gradually during the experimental period, and BCS in the MF group was significantly lower 1 month after calving compared to the Control group (Figure 1). Markedly reduced DMI and BCS were reported in dairy cows with milk fever after calving [26-28]. Therefore, decreased FAA including tryptophan in the MF Group may be associated with a more pronounced decrease in DMI in the MF cows.

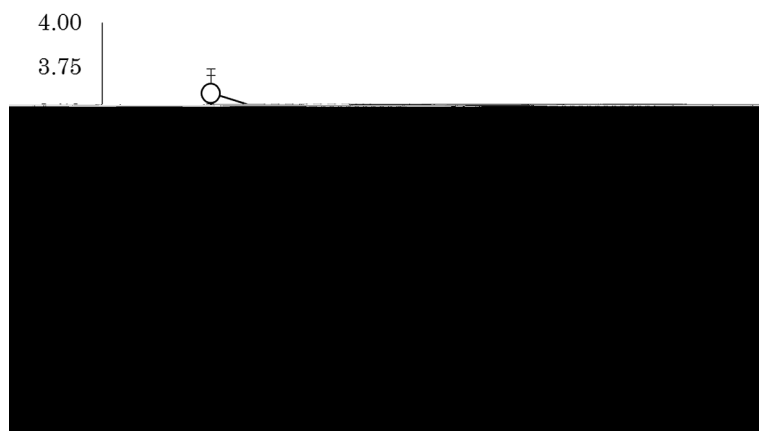


Figure 1. Changes of BCS score in two groups. Black circle represents MF Group (N=7), and white circle represents Control Group (N=16). Results are expressed as the mean ($\pm$ SE). Asterisks indicate significant differences between the two groups ($P < 0.05$).

Serum cholesterol levels in both groups decreased on the day of calving and recovered after that. Conversely, serum NEFA levels increased in two groups on the day of calving. There was no significant difference in these parameters. BHB levels in both groups continued to increase during this observation period and a significantly lower level in the MF group than the Control group was observed at 1 month (Table 5).

The lactating cow with above 1.2 mmol/l of serum BHB level is diagnosed as subclinical ketosis [29], and strong correlations among blood NEFA and BHB levels and energy balance in early lactation dairy cows have been reported [30]. Hyperketonemia was suggested as one of the most frequent findings in high producing dairy cows.

In conclusion, MF cows showed lower levels in several FAA around the time of calving. Proteins stored in the maternal muscles are released to augment the supply of amino acids for fetal growth and development during the later pregnancy. Zhang et al [31] described that maternal nutrition during gestation has a marked effect on the muscle, bone and the other organs development of cattle fetuses. The cows with undernutrition, such as FAA and cholesterol, might be at risk of decreased muscle function due to demands of amino acids for the fetus before calving. This study did not clarify the effect of low serum FAA and cholesterol during the dry period on the onset of milk fever, or whether low FAA directly affected calcium metabolism. Further

research is required to determine the specific reasons for the various differences in serum FAA and lipids in the MF cows around the time of calving.

Table 5. Serum free non-essential amino acid concentration in the tested groups

		MF Group	Control Group
TC (mg/dL)	Dry	131.57±11.64	121.50±5.10
	after calving	71.86±6.09	68.6875±3.79
	1 Month	165.43±7.91	181.38±7.36
NEFA (μEq/L)	Dry	0.13±0.03	0.10±0.01
	after calving	0.90±0.19	0.71±0.05
	1 Month	0.29±0.05	0.39±0.05
BHB (μmol/L)	Dry	622.14±29.41	672.00±36.15
	after calving	1023.29±129.36	798.94±49.51
	1 Month	1348.00±167.98	2433.50±249.54 *

Data are expressed as mean ± SE. Astarisk indicate significant difference between the two tested groups ($P < 0.05$). **TC**=Total cholesterol, **NEFA**=Non-esterified Fatty Acid, **BHB**=β-hydroxybutyric acid.

Authors' contributions

KK participated in the design of the study, carried out the sampling of blood, performed the statistical analysis, and drafted the manuscript. HO carried out the assays of amino acid in the serum, conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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NIŽI NIVO SLOBODNIH AMINOKISELINA U SERUMU KOD HOLŠTAJN KRAVA SA MLEČNOM GROZNICOM – SLUČAJ SA TERENA

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Ova studija je sprovedena radi ispitivanja veze između slobodnih aminokiselina (FAA) u serumu i pojave mlečne groznice kod mlečnih krava u vreme teljenja. Serumska kon-

centracija FAA je upoređena kod multiparnih krava sa mlečnom groznicom (MF grupa; n=7) i zdravih multipara (kontrolna grupa; N=16) koje se drže na farmi mlečnih Holštajn krava. Uzorci krvi su uzeti 3 do 4 nedelje pre teljenja, na dan teljenja i 1 mesec nakon teljenja radi analize aminokiselina u serumu i parametara lipida. Nivoi svih esencijalnih aminokiselina u MF grupi bili su niži nego u kontrolnoj grupi. Značajne razlike između grupa primećene su u fenilalaninu, valinu, izoleucinu i leucinu 1 mesec nakon teljenja, i u triptofanu na dan teljenja. Što se tiče neesencijalnih aminokiselina, značajno niži nivo arginina primećen je u MF grupi u poređenju sa kontrolnom grupom na dan teljenja, kao i 1 mesec nakon teljenja. Značajna pozitivna korelacija je otkrivena između nivoa holesterola i nivoa aminokiselina razgranatog lanca (BCAA) ($r=0,531$, $P<0,01$). Ovi rezultati su pokazali da su MF krave imale niže nivoe nekoliko FAA oko vremena teljenja, čak i pre početka MF.