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Original article

RELATIONSHIPS AMONG CIRCULATING METABOLIC BIOMARKERS IN HEALTHY HIGH-PRODUCING HOLSTEIN DAIRY COWS IN DIFFERENT PHYSIOLOGICAL STATES

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Summary

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Information regarding metabolic profile in different physiological sates of dairy cows can assist veterinarians to monitor the herd health and productive performance. Furthermore, the relationships among the metabolic parameters can be used to detect the effect of each parameter on another one. The aim of the current research was to clarify the interactions among metabolic parameters in different physiological states of high producing Holstein dairy cows. The present study was carried out on 25 multiparous Holstein dairy cows divided into 5 equal groups: early, mid and late lactation; far-off and close-up dry. Blood samples were collected from all cows through jugular venipuncture and sera were separated to evaluate glucose, insulin, β-hydroxybutyric acid (BHBA), non-esterified fatty acids (NEFA), triglyceride, cholesterol, high-density lipoprotein, low-density lipoprotein and very low-density lipoprotein. There were negative and significant correlations between insulin and glucose in all studied groups ($P<0.05$). The correlation coefficients among insulin and both NEFA and BHBA in all cows were negative. In all studied groups, the negative correlations were seen among glucose and both NEFA and BHBA. In all studied groups, glucose and insulin were negatively correlated with lipid profile. The correlation between BHBA and NEFA was positive, strong and significant in all groups ($P<0.05$). In all studied groups, BHBA and NEFA were positively correlated with lipid profile but no significant correlations were seen among them ($P>0.05$). The correlations among studied metabolic parameters showed that providing the energy demands can prevent the ketogenic and lipolytic metabolisms in high producing dairy cows. Furthermore, information regarding the correlations among circulating metabolic parameters can be used to estimate the changing patterns of each metabolic parameter via evaluating another one.

Key words: correlation, energy demands, metabolic biomarkers, Holstein dairy cows

INTRODUCTION

Dairy cows undergo metabolic changes during pregnancy and lactation (Tanritanir *et al.,* 2009). Each state has metabolic characteristics which are different from those of others. Two to three weeks before parturition, a phase of catabolism starts to

prepare the cow for parturition and the initiation of lactation. Immediately after the calving, high rates of body condition score losses are associated with a severe negative energy balance status, indicated by alterations in blood metabolite and hormone profiles (Wathes *et al.,* 2009). The negative energy balance will last during the lactation period depending on the feeding and management strategies and the genetic potential of the animal. The last period of the production year is a phase of anabolism where the emphasis is put on increased weight gain as a long-term preparation for the next lactation (Piccione *et al.,* 2012).

The pathogenesis of metabolic diseases such as fatty liver and ketosis is closely linked to the first weeks of lactation when increases in feed intake lag behind increases in milk production (Bobe *et al.,* 2004). The concomitant negative energy balance tends to be more pronounced in high yielding dairy cows than in substandard animals. The negative energy balance represents a distinct risk factor for metabolic disturbances. These imbalances can induce different alterations in circulating metabolic profile (Radostits *et al.,* 2007). Information regarding the metabolic parameters in each physiological state can aid veterinarians to make diagnosis and prognosis of metabolic disorders in high producing dairy cows (Duffield *et al.,* 2000).

There are several studies on metabolic profile during the periods of the productive life of the cows such as transition and lactation periods (Ghanem *et al.,* 2012; Piccione *et al.,* 2012; Fiore *et al.,* 2014). Based on author's knowledge, there is little information regarding the circulating metabolic profile in different physiological states of dairy cows in a comprehensive study. Therefore, the present study was designed to clarify the metabolic profile in high producing Holstein dairy cows in early, mid and late lactation and far-off and close-up dry periods. Furthermore, the relationships among these parameters together can represent the effect of each parameter on other ones in each physiological state.

MATERIALS AND METHODS

Animals

The present study was carried out during the winter of 2014 on 25 Holstein dairy cows, with three parturitions, from a high producing industrial dairy farm around Shiraz, Southwest Iran. These cows were housed in open-shed barns with free access to water and shade. The total mixed rations were formulated and prepared for all animals according to National Research Council (NRC) requirements (Table 1). At this farm, a dry period of 60 days has been considered. Milk production was about 10,000 kg for year, average milk fat 3.6%, and milk protein – 3.3%. All animals were clinically healthy, had no history of debilitating disease, and were free from internal and external parasites due to routine antiparasitic programmes at this farm. Body condition scores (BCS) of animals were estimated based on 0 to 5 system. Cattle were divided to 5 equal groups containing early (30.2±5.7 days after calving, with 3.25±0.25 BCS and 48.2±4.4 kg milk production), mid (108.1±8.4 days after calving, with 3.25±0.25 BCS and 51.5±6.2 kg milk) and late lactations (184.5±5.7 days after calving, with 3.5±0.25 BCS and 30.2±2.8 kg milk), far-off (281.9±5.4 days after calving, 228.4±8.6 days of pregnancy, with 3.5±0.25 BCS) and close-up dry periods (312.1±8.3 days after calving,

Table 1. The basic nutrient content of rations in different physiological states of studied high-producing Holstein dairy cows

Nutrient	Early lactation	Mid lactation	Late lactation	Far-off dry	Close-up dry
DMI (kg/day)	30	24	20	14	10
NEL (Mcal/kg)	1.61	1.47	1.36	1.32	1.43
Fat (%)	7	6	4	3	4
CP (%)	16.7	15.2	14.1	9.9	12.4
RDP (%)	9.8	9.7	9.5	7.7	9.6
RUP (%)	6.9	5.5	4.6	2.2	2.8
MP (%)	11.6	10.2	9.2	6.0	8.0
NDF (%)	28	30	32	40	35
ADF (%)	19	21	24	30	25
NFC (%)	38	35	32	30	34
Calcium (%)	0.60	0.61	0.62	0.44	0.48
Phosphorus (%)	0.38	0.35	0.32	0.22	0.26
Magnesium (%)	0.21	0.19	0.18	0.11	0.40
Chlorine (%)	0.29	0.26	0.24	0.13	0.20
Sodium (%)	0.22	0.23	0.22	0.10	0.14
Potassium (%)	1.07	1.04	1.00	0.51	0.62
Sulfur (%)	0.20	0.20	0.20	0.20	0.20
Vitamin A (IU/day)	75,000	75,000	75,000	80,300	83,270
Vitamin D (IU/day)	21,000	21,000	21,000	21,900	22,700
Vitamin E (IU/day)	545	545	545	1,168	1,200

DMI: dry matter intake; NEL: net energy lactation; CP: crude protein; RDP: rumen degradable protein; RUP: rumen undegraded protein; MP: metabolisable protein; ME: metabolisable energy; NDF: neutral detergent fibre; ADF: acid detergent fibre; NFC: non-fibre carbohydrate. Trace mineral added to ration (ppm): cobalt: 0.11; copper 10–18; iodine: 0.3–0.4; iron: 13–130; manganese: 14–24; selenium: 0.30 and zinc: 22–70.

255.6±6.3 days of pregnancy, with 3.5±0.25 BCS).

Blood sampling and serological assays

Blood samples were collected from all cows through jugular venipuncture in plain tubes. Immediately after blood collections, sera were separated by centrifugation for 10 min at 3,000g and stored at –22 ^{o}C until assayed. Glucose was assayed by an enzymatic (glucose oxidase) colorimetric method (ZistChem®, Tehran, Iran). Insulin was measured by bovine insulin ELISA kit (Cusabio®, China, specificity 100%, and precision: intra-assay and inter-assay CV < 8% and 10%, respectively). Non-esterified fatty acids (NEFA) and β-hydroxybutyric acid (BHBA) were assayed by colorimetric methods (Ranbut®, Ireland). The sera were analysed for cholesterol by a modified Abell-Kendall/Levey-Brodie (A-K) method (Abbel *et al.,* 1952; Burtis & Ashwood, 1994), triglycerides (TG) by the enzymatic procedure of McGowan *et al.* (1983). Lipoproteins were isolated using a combination of precipitation and ultra centrifugation. High-density lipoprotein (HDL) cholesterol was measured using the precipitation method. In the first step, the precipitation reagent (sodium phosphotungstate with magnesium chloride)

was added to the serum to aggregate non-HDL lipoproteins which were sedimented by centrifugation (10,000×g for 5 min). The residual cholesterol was then measured by an enzymatic method (Burtis & Ashwood, 1994). Low-density lipoprotein (LDL) cholesterol was calculated as the difference between the total cholesterol measured in the precipitate and in the HDL fraction minus 0.2×triglyceride (LDL=total cholesterol–HDL cholesterol–0.2×TG). Very low-density lipoprotein (VLDL)-cholesterol was estimated as one-fifth of the concentration of triglycerides (Friedewald *et al.,* 1972).

Statistical analyses

The normal values of metabolic parameters in each physiological state are presented as mean±SD. Differences between the average concentrations of different serological factors in the different groups were analysed by one-way ANOVA and the least significant difference (LSD) test to evaluate differences. Pearson's correlation test was used to detect the relationship among studied parameters at each physiologic state, separately, using SPSS software (SPSS for Windows, version 20, SPSS Inc, Chicago, IL, USA). In the present study, the Pearson's correlation coefficient greater than 0.8 was considered as strong, whereas, a coefficient lesser than 0.5 described as weak. The level of significance was set at $P<0.05$.

RESULTS

Normal levels (mean±SD) of circulating metabolic biomarkers in different physiological states of high producing Holstein

Table 2. Levels of circulating metab olic biomarkers (mean±SD; n=5 in each group) of high producing Holstein dairy cows in different physiological states

Biomarkers	Early lactation	Mid lactation	Late lactation	Far-off dry	Close-up dry
Glucose (mmol/L)	4.93±1.08[a]	4.17±0.61[a]	5.10±2.81[a]	4.47±2.81[a]	5.02±1.92[a]
Insulin (pmol/L)	201.9±0.3[a]	207.0±4.5[b]	203.7±2.4[a]	201.8±1.6[a]	207.4±3.8[b]
NEFA (mmol/L)	0.34±0.01[a]	0.31±0.01[a]	0.24±0.01[b]	0.24±0.01[b]	0.29±0.01[a]
BHBA (mmol/L)	88.58±3.57[a]	79.71±4.78[a]	72.42±4.24[b]	64.17±4.23[b]	82.79±2.97[a]
Cholesterol (mmol/L)	3.96±0.83[a]	5.04±0.66[b]	4.92±1.01[b]	4.02±0.76[a]	3.76±0.63[a]
Triglycerides (mmol/L)	1.39±0.25[a]	1.19±0.10[a]	1.25±0.12[a]	1.30±0.06[a]	1.28±0.24[a]
HDL (mmol/L)	2.61±0.57[a]	2.76±0.47[a]	2.90±0.40[a]	3.02±0.37[a]	2.53±0.47[a]
LDL (mmol/L)	1.49±0.44[a]	2.16±0.49[b]	0.86±0.51[c]	0.84±0.44[c]	1.29±0.38[a]
VLDL (mmol/L)	0.27±0.05[a]	0.23±0.02[a]	0.25±0.02[a]	0.26±0.01[a]	0.25±0.04[a]

[a,b,c] Different letters indicate significant differences in each row ($P<0.05$). NEFA= non-esterified fatty acids; BHBA=β-hydroxybutyric acid; HDL=high-density lipoprotein; LDL= low-density lipoprotein; VLDL= very low-density lipoprotein

dairy cows are presented in Table 2. Insulin levels in mid lactation and close-up dry cows were significantly higher than other groups ($P<0.05$) and the lowest insulin concentration was detected in far-off dry group. Serum concentrations of NEFA and BHBA in early and mid lactation and close-up dry cows were significantly higher than late lactation and far-off dry animals ($P<0.05$). There were no significant differences between late lactation and far-off dry groups. Baseline levels of cholesterol in mid and late lactation were significantly higher than other groups. The level of LDL in mid lactation cows was significantly higher than in others, and its value in far-off dry cows was significantly lower than other groups ($P<0.05$). The baseline values of glucose, TG, HDL and VLDL did not differ significantly among all studied animals ($P>0.05$; Table 2).

The correlations among circulating metabolic parameters in studied dairy cows at different physiological states are presented in Tables 3 to 7. There were negative and significant correlations between insulin and glucose in all studied groups ($P<0.05$; Tables 3 to 7). The correlation coefficients among insulin and both NEFA and BHBA in all cows were negative. These negative correlations were significant in close-up dry cows ($P<0.05$; Table 7). In all studied groups, the negative correlations were seen among glucose and both NEFA and BHBA. Furthermore, these negative correlations were strong and significant in early lactation cows ($P<0.05$; Table 3). In all studied groups, glucose and insulin were negatively correlated with lipid profile (cholesterol, TG, HDL, LDL and VLDL) but in close-up dry cows, the negative correlations were strong and significant among glucose and lipid profile ($P<0.05$; Table 7). The correlation between BHBA and NEFA was positive, strong and significant in all groups ($P<0.05$; Tables 3 to 7). In all studied groups, BHBA and NEFA were positively correlated with lipid profile (cholesterol, TG, HDL, LDL and VLDL) but no significant correlations were seen among them ($P>0.05$; Tables 3 to 7). TG had positive, strong and significant correlation to VLDL in all animals ($R=1.000$; $P<0.05$; Tables 3 to 7).

DISCUSSION

Lactogenesis around parturition and rapidly increasing milk production after calving greatly increase demands for glucose to milk lactose synthesis, at a time when feed intake has not reached its maximum (Goff & Horst, 1997). In dairy cows little glucose is absorbed directly from the digestive tract because much of the dietary carbohydrate is fermented in the rumen to produce volatile fatty acids such as propionate. Consequently, dairy cows rely almost exclusively on gluconeogenesis (synthesis of glucose) from propionate in the liver to meet their glucose requirements. Limited feed intake during the early lactation means that supply of propionate for glucose synthesis also is limited (Drackley *et al.,* 2001). Despite the need of energy supply to high milk yield, the dry matter intake is lower than energy demands and a negative energy balance takes place in the lactation period (Radostits *et al.,* 2001). Negative energy balance can induce metabolic dysfunctions in dairy cows, hence the information regarding metabolic changes and correlations during the different physiological states of high producing dairy cows can aid veterinarians to diagnose and prevent metabolic disorders.

The significant negative correlations between glucose and insulin in this study

Table 3. Correlations among circulating metabolic parameters in high producing Holstein dairy cows (n=5) at early lactation period

	Insulin	Glucose	NEFA	BHBA	Cholesterol	Triglycerides	HDL	LDL	VLDL
Glucose	–0.868*								
NEFA	–0.218	–0.858*							
BHBA	–0.147	–0.894*	0.817*						
Cholesterol	–0.468	–0.118	0.485	0.083					
Triglycerides	–0.527	–0.355	0.642	0.449	–0.214				
HDL	–0.190	–0.329	0.176	0.047	–0.252	–0.414			
LDL	–0.533	–0.482	0.138	0.365	0.793	–0.314	–0.567		
VLDL	–0.527	–0.355	0.642	0.449	–0.214	1.000*	–0.414	–0.314	
Milk yield	0.746*	–0.243	–0.564	–0.752*	0.665	0.335	0.023	0.345	0.012

*statistically significant correlations at P<0.05; NEFA= non-esterified fatty acids; BHBA=β-hydroxybutyric acid; HDL=high-density lipoprotein; LDL= low-density lipoprotein; VLDL= very low-density lipoprotein.

Table 4. Correlations among circulating metabolic parameters in high producing Holstein dairy cows (n=5) at mid lactation period

	Insulin	Glucose	NEFA	BHBA	Cholesterol	Triglycerides	HDL	LDL	VLDL
Glucose	–0.797*								
NEFA	–0.389	–0.386							
BHBA	–0.361	–0.407	0.954*						
Cholesterol	–0.231	–0.376	0.113	0.026					
Triglycerides	–0.665	–0.467	0.119	0.013	0.006				
HDL	–0.147	–0.428	0.606	0.784	–0.067	–0.113			
LDL	–0.237	–0.425	0.736	0.783	0.537	0.344	–0.610		
VLDL	–0.665	–0.467	0.119	0.013	0.006	1.000*	–0.113	0.344	
Milk yield	0.842*	–0.156	–0.642*	–0.432	0.723*	0.571	0.117	0.411	0.032

*statistically significant correlations at P<0.05; NEFA= non-esterified fatty acids; BHBA=β-hydroxybutyric acid; HDL=high-density lipoprotein; LDL= low-density lipoprotein; VLDL= very low-density lipoprotein.

Table 5. Correlations among circulating metabolic parameters in high producing Holstein dairy cows (n=5) at late lactation period

	Insulin	Glucose	NEFA	BHBA	Cholesterol	Triglycerides	HDL	LDL	VLDL
Glucose	–0.782*								
NEFA	–0.767	–0.362							
BHBA	–0.298	–0.259	0.850*						
Cholesterol	–0.200	–0.193	0.181	0.275					
Triglycerides	–0.047	–0.638	0.427	0.058	0.126				
HDL	–0.688	–0.528	0.299	0.028	–0.115	–0.257			
LDL	–0.107	–0.335	0.096	0.546	–0.695	–0.592	–0.080		
VLDL	–0.562	–0.638	0.427	0.058	0.150	1.000*	0.450	–0.661	
Milk yield	0.212	–0.032	0.046	0.111	0.046	0.198	0.044	0.028	0.091

*statistically significant correlations at $P<0.05$; NEFA= non-esterified fatty acids; BHBA=β-hydroxybutyric acid; HDL=high-density lipoprotein; LDL= low-density lipoprotein; VLDL= very low-density lipoprotein.

Table 6. Correlations among circulating metabolic parameters in high producing Holstein dairy cows (n=5) at far-off dry period

	Insulin	Glucose	NEFA	BHBA	Cholesterol	Triglycerides	HDL	LDL
Glucose	–0.834*							
NEFA	–0.542	–0.079						
BHBA	–0.344	–0.263	0.851*					
Cholesterol	–0.367	–0.653	0.113	0.543				
Triglycerides	–0.063	–0.010	0.382	0.657	–0.728			
HDL	–0.534	–0.424	0.735	0.607	0.750	–0.800		
LDL	–0.147	–0.089	0.114	0.522	–0.812	0.957*	–0.677	
VLDL	–0.063	–0.010	0.382	0.657	–0.728	1.000*	–0.800	0.957*

*statistically significant correlations at P<0.05; NEFA= non-esterified fatty acids; BHBA=β-hydroxybutyric acid; HDL=high-density lipoprotein; LDL= low-density lipoprotein; VLDL= very low-density lipoprotein.

Table 7. Correlations among circulating metabolic parameters in high producing Holstein dairy cows (n=5) at close-up dry period

	Insulin	Glucose	NEFA	BHBA	Cholesterol	Triglycerides	HDL	LDL
Glucose	–0.832*							
NEFA	–0.928*	–0.298						
BHBA	–0.980*	–0.151	0.977*					
Cholesterol	–0.260	–0.936*	0.050	0.105				
Triglycerides	–0.627	–0.820*	0.547	0.535	0.310			
HDL	–0.009	–0.919*	0.358	0.182	0.795	0.226		
LDL	–0.118	–0.932*	0.249	0.056	0.913*	0.136	0.948*	
VLDL	–0.627	–0.820*	0.547	0.535	0.310	1.000*	0.226	0.136

*statistically significant correlations at P<0.05; NEFA= non-esterified fatty acids; BHBA=β-hydroxybutyric acid; HDL=high-density lipoprotein; LDL= low-density lipoprotein; VLDL= very low-density lipoprotein.

can be explained as the effects of insulin on the clearance of circulating glucose. Insulin and glucose were negatively correlated to BHBA and NEFA in all studied animals. It can be suggested that the high levels of glucose and insulin can prevent the synthesis of BHBA and NEFA via supplying and utilising the energy demands. In a negative energy balance situation, released fatty acids from adipose tissue circulate as NEFA, which are a major source of energy to the cow (De Koster & Opsomer, 2013). The concentration of NEFA in blood reflects the degree of adipose tissue mobilisation (Pullen *et al.* 1989); therefore, as negative energy balance increases, more NEFA are released from body fat and the concentration of NEFA in blood increases. As the concentration of NEFA in blood increases around calving or in early lactation, more NEFA are taken up by the liver (Emery *et al.,* 1992). Once taken up by the liver, NEFA can be completely oxidised to carbon dioxide to provide energy for the liver, partially oxidised to produce ketone bodies such as BHBA that are released into the blood and serve as fuels for other tissues, or reconverted to storage TG (De Koster & Opsomer, 2013).

There were negative correlations among insulin and glucose with lipid profile, i.e. the high levels of insulin and glucose can provide the energy demands and prevent the lipolytic effects of negative energy balance. Decreasing the lipogenic activity in adipose tissue takes place during negative energy balance. While the overall effect of decreasing lipogenic activity of adipose tissue is to supply milk fat precursors to the mammary gland, there are additional negative consequences on tissues throughout the body. Primarily, the liver is affected if uptake of fatty acids exceeds its ability to oxidise them or export them in the form of VLDL (Goff & Horst, 1997). Cows given free access to feed during the dry period have as much as two-fold higher postpartum hepatic TGs (Van den Top *et al.,* 1996). The resulting accumulation of TGs in the liver, known as fatty liver, decreases the gluconeogenic capacity (Goff & Horst, 1997). If the liver has decreased ability to make glucose to support milk production, this further extends the amount of lipolysis which must occur in order to support lactation (Rukkwamsuk *et al.,* 1999).

The positive correlations between BHBA and NEFA can be explained as the increase of plasma NEFA concentration led to the increase of ketogenesis by hepatocytes (Grummer *et al.,* 2004). Fiore *et al.* (2014) demonstrated that glucose infusion influenced metabolism in ruminants modifying glucose, insulin, NEFA and BHB profile. They presented that glucose is an important direct controller of metabolic interactions and responses in dairy cows. The decrease of glucose and the increase of NEFA and BHBA in their study, explains our results about negative correlations among glucose and insulin and both BHBA and NEFA concentrations.

In conclusion, the results of the present study provide the normal value of metabolic profile in different physiological states of high producing Holstein dairy cows. These values can assist veterinarians to diagnose metabolic abnormalities by determining circulating metabolic parameters. Evaluating the circulating metabolic biomarkers in dairy cows and compare them to normal reference values presented in this research can discover the metabolic abnormalities in the herd and assist veterinarians and farmers to better manage the metabolic problems at the herd level. The correlations among stu-

died metabolic parameters showed that meeting the energy demands can prevent the ketogenic and lipolytic metabolisms in high producing dairy cows. Finally, information regarding the correlations among circulating metabolic parameters can be used to estimate the changing patterns of each metabolic parameter by evaluating another one.

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