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Short Paper

A field study on glucose, non-esterified fatty acids, beta-hydroxybutyrate and thyroid hormones in dairy cows during the breeding period in Fars province, Iran

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Summary

Background: It was hypothesized that under apparently good management practices in dairy farms, some cows may not be metabolically perfect during the breeding period and this may affect their performance. **Aims:** This study was conducted to assess probable metabolic drawbacks in mid-lactation dairy cows affecting their performance. **Methods:** Thirty-seven clinically healthy Holstein cows were assessed for plasma concentrations of glucose, non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), thyroxin (T4), and tri-iodothyronine (T3) on days 60, 90 and 120 of lactation. The relationships of the measured analytes with some performance indices were also studied. **Results:** Continuous declines in plasma glucose (within reference values; $P<0.006$), T4 ($P<0.001$) and T3 (0.003) were found during the study. Non-esterified fatty acids showed relatively high levels through the study with a rise at day 90 ($P<0.041$). Beta-hydroxybutyrate concentrations did not change significantly ($P>0.05$) but were higher than those reported by others in mid-lactation cows. By progress in lactation 27% of cows had glucose concentrations <2.5 mmol/L, 62% had NEFA concentrations >0.40 mmol/L and 13.5% had BHB levels above 1200 µmol/L, which are the threshold levels of peri- and post-parturient problems. Milk production had negative correlations with glucose and T4 while the correlation was positive with NEFA. The interval between calving to the 1st heat had positive correlations with BHB concentrations. The interval between calving and the 1st insemination was inversely correlated with glucose and positively correlated with NEFA levels. **Conclusion:** The changes of the studied analytes in mid-lactation cows resembled those that would happen during negative energy balance in early lactation cows and feed restriction in mid-lactation cows and may affect the general health and the performance of the cows.

Key words: Breeding period, Dairy cows, Glucose, NEFA, Thyroid hormones

Introduction

In accordance with the targets of dairy industry, many Iranian dairy farms anticipate that the high producing cows persist on high milk production and become pregnant as soon as possible during the breeding period (first insemination at 67 ± 38 days; Ansari-Lari *et al*., 2010). Generally, it is believed that the cows will perform well during the breeding period (mid-lactation as a whole) with optimized management and nutrition and minimizing multiple stressors during the preceding periods of the lactation cycle (Drackley, 1999, 2006; Overton, 2004; Varga, 2004, 2005; Bertoni and Trevisi, 2013; Lucy, 2016). Despite apparently good management routines, many Iranian farms have extended days open (134 ± 89; Ansari-Lari *et al*., 2010), prolonged calving intervals (413 ± 81 days; Atashi *et al*., 2012) and high rates of culling for reproductive failure (>30% of culls vs. <25% in western countries; Mohammadi and Sedighi, 2009; Ansari Lari *et al*., 2012; Rushen and de Passillé, 2013).

Based on field experiences, nearly all inseminations in Fars province are performed based on clinical findings combined with routine reproductive hormonal protocols, regardless of the actual metabolic status of the cows. Mild stressful and inflammatory conditions leading to acute phase response may happen during both stages of early- and mid-lactation (Mohebbi-Fani *et al*., 2016). It was hypothesized that, under apparently good management systems in dairy farms, high producing cows may not be metabolically perfect during the breeding period. The plasma concentrations of glucose, non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB) and thyroid hormones of Holstein cows during days 60 to 120 of lactation were used to assess this hypothesis. Milk production and reproductive indices of the cows were also assessed.

Materials and Methods

The study was conducted during late summer and early autumn in a Holstein farm, holding 200 milking cows, 60 km north of Shiraz, Fars province, Iran. The lactating cows were kept in wall-less free stalls (10-15% less than the actual barn capacity) with sand beds, concrete walking areas and free access to feed and water. The 1st lactation cows had their own separate group. All freshening cows were held in a fresh pen for 2-3 weeks post-calving. Both early- and mid-lactation cows were fed the same total mixer ration (TMR) (Table 1; formulated based on printed feed composition tables) *ad libitum* with 2-4% daily feed refusals. The feeding practice was unchanged from about 2 months before the study and during the sampling period (3 months). Veterinary supervisions were performed biweekly and all cows received the postpartum examinations and treatments and were inseminated after day 60 postpartum. The cows were milked two times a day at 0500 and 1700 and their milk production was recorded monthly.

Table 1: The ingredients and chemical composition of the cows' rations during early- and mid-lactation

Ingredients (DM basis)	
Alfalfa hay (%)	17.32
Corn silage (%)	25.91
Barley (ground) (%)	16.33
Corn (ground) (%)	16.10
Wheat bran (%)	5.90
Soybean meal (%)	11.23
Meat and bone meal (poultry) (%)	4.74
Mineral supplement (%)	0.62
Vitamin supplement (%)	0.31
Calcium carbonate (%)	0.31
Salt (white) (%)	0.31
Sodium bicarbonate (%)	0.94
Monensin (mg/cow)	360-400 (1st calf heifers and adults, respectively)
Chemical composition *	
DM (kg)	22.3-25.9 (1st calf heifers and adults, respectively)
fNDF (% DM)	21.4
NDF (% DM)	30.6
NEl (mcal/kg)	1.56
CP (% DM)	17.80
MP (% DM)	10.68
RUP (% CP)	33.8
EE (% DM)	3.02
NFC (% DM)	42.8
Starch (% DM)	28.02
Ca (% DM)	0.96
P (% DM)	0.63
Ca:P ratio (DM)	1.52
Forage ratio (DM)	43.23

* Calculated (Spartan ration evaluator/balancer for dairy cattle, version 3.0.3). DM: Dry matter, fNDF: Forage NDF, NDF: Neutral detergent fiber, NEl: Net energy for lactation, CP: Crude protein, MP: Metabolizeable protein, RUP: Rumen undegradable protein, EE: Ether extract, NFC: Non-fibrous carbohydrates, Ca: Calcium, and P: Phosphorus

Among >40 post-parturient cows, 20 primiparous and 17 multiparous clinically healthy cows were enrolled in the study from day 60 postpartum. Whole blood samples (10 ml in EDTA tubes) were drained from coccygeal vessels on days 60, 90 and 120 (±5 days) of lactation under excellent restraint conditions (the instructions of the committee on animal ethics, Shiraz University, Shiraz, Iran). The samples were taken in the morning between 0900 and 1030. The body condition score and locomotion score of each cow were recorded after exiting from the restraint pen. The performance data of the whole lactation were obtained from the database of the farm.

Within 3 h of the first sampling the specimens were centrifuged (3000 g, 10 min) and plasmas were stored at -20°C. Glucose was assayed by oxidase colorimetric method (ZistChem®, Tehran, Iran). Non-esterified fatty acids were measured by biotin double antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Bioassay Technology Laboratory, Shanghai, China). Bata-hydroxybutyrate was assessed by colorimetric method (Ranbut®, Randox Com., Ireland). Thyroxine (T4) and tri-iodothyronine (T3) were measured using competitive enzyme immunoassay kits (Monobind Inc., CA, USA). The intra- and inter-assay CVs were 3.0% and 3.7% for T4 and 12.6% and 13.2% for T3. The sensitivity of the tests for T4 and T3 was 0.4 mg/dL and 0.2 ng/ml, respectively.

The data (mean±SD) were analyzed using SPSS statistical software (version 16.0, SPSS Inc., Chicago, IL, USA) at $P \leq 0.05$. The trends of changes during days 60 to 120 of lactation were studied in all cows, and were compared between primiparous and multiparous cows using analysis of variance (ANOVA) for repeated measures. The plasma concentrations of glucose, NEFA and BHB of each animal at each sampling day were compared with published cut-off points used for assessing sub-clinical ketosis and fat mobilization in periparturient cows. The correlations between the measured analytes and those between the analytes and milk production (sum of milk of days 60, 90 and 120; 305-day milk) and reproductive indices (calving to 1st heat; calving to 1st insemination; service per conception; days open) were assessed using Pearson's correlation test.

Results

A continuous decrease in plasma glucose ($P=0.006$), T4 ($P<0.001$) and T3 ($P=0.003$) was found through days 60 to 120 of lactation (Table 2). Non-esterified fatty acids showed a quadratic pattern of change: a rise at day

Table 2: Trends of changes of the studied plasma parameters from day 60 to day 120 of lactation

Plasma parameters	Day 60	Day 90	Day 120	P-value
Glucose (mmol/L)	3.60 ± 0.79	3.42 ± 0.87	3.11 ± 0.87	0.006
NEFA (mmol/L)	0.35 ± 0.14	0.41 ± 0.14	0.38 ± 0.16	0.041 (Q)
BHB (µmol/L)	814.68 ± 483.14	843.62 ± 459.48	889.96 ± 463.40	0.45
T4 (nmol/L)	37.82 ± 9.47	34.92 ± 7.17	31.08 ± 8.93	0.001
T3 (nmol/L)	1.78 ± 0.68	1.74 ± 0.70	1.39 ± 0.35	0.003

NEFA: Non-esterified fatty acids, BHB: Beta-hydroxybutyrate, T4: Thyroxine, T3: Tri-iodothyronine, and Q: Quadratic change

Table 3: The number (%) of cows showing low glucose and high NEFA and BHB concentrations at various sampling days

Plasma parameters	Day 60	Day 90	Day 120	Alarm level [*]
Glucose <2.5 mmol/L	2 (5.4%)	5 (13.5%)	10 (27%)	
NEFA >0.4 mmol/L	15 (40%)	23 (62%)	17 (46%)	>10%
BHB >1200 µmol/L	4 (11%)	5 (13.5%)	5 (13.5%)	>10%

NEFA: Non-esterified fatty acids, BHB: Beta-hydroxybutyrate, and [*] periparturient cows

Table 4: Observed correlation between the studied plasma parameters on days 60, 90 and 120 of lactation

Day 60-120	Day 60	Day 90
NEFA × T4 (r=-0.45; P=0.007)	NEFA × T4 (r=-0.44; P=0.008)	
Glucose × NEFA (r=-0.303; P=0.081)	Glucose × T4 (r=0.29; P=0.084)	Glucose × T3 (r=0.32; P=0.054)

NEFA: Non-esterified fatty acids, T4: Thyroxine, and T3: Tri-iodothyronine

Table 5: Observed correlations between the performance indices and the mean of the studied plasma parameters during the entire period of the study (days 60 to 120 of lactation)

Plasma parameters	Milk 60-120	Milk 305 day	Calving-1st heat	Calving-1st insemination
Glucose	r=-0.353 (P=0.037)	r=-0.347 (P=0.041)		r=-0.298 (P=0.082)
NEFA	r=0.393 (P=0.02)	r=0.392 (P=0.02)		r=0.423 (P=0.011)
BHB			r=0.394 (P=0.016)	
T4	r=-0.431 (P=0.008)	r=-0.423 (P=0.009)		
T3			r=0.465 (P=0.004)	r=0.277 (P=0.097)

NEFA: Non-esterified fatty acids, BHB: Beta-hydroxybutyrate, T4: Thyroxine, and T3: Tri-iodothyronine

90 and then a decrease at day 120 (P=0.041). Beta-hydroxybutyrate increased through the study but its changes were not significant (P>0.05). The trends of changes were not different between primiparous and multiparous cows.

Low glucose concentrations or high NEFA and BHB levels (compared with the cut-off points of sub-clinical ketosis or high body fat mobilization) were observed in some cows at each sampling day (Table 3) without clinical signs of ketosis.

Non-esterified fatty acids and T4 showed inverse correlations at day 60 of lactation (r=-0.44, P=0.008; Table 4) and during the entire period of the study (r=-0.45, P=0.007). Glucose also showed a negative correlation with NEFA of the entire period of the study, and positive correlations with T4 of day 60 and T3 of day 90 that tended to be significant (P<0.1).

Milk production had negative correlations with glucose and T4 and positive relationship with NEFA (Table 5). The interval between calving to 1st heat had positive correlations with BHB and T3 concentrations. The interval between calving and the 1st insemination was inversely correlated with glucose and positively correlated with NEFA and T3 levels. Service per conception and days open had no correlations with the studied analytes.

Discussion

It is generally believed that the metabolic profile of dairy cows after 30 days postpartum still involves relatively low concentrations of glucose, but normal concentrations of NEFA and BHB (Lucy *et al*., 2014). In the present study, the changes of the studied analytes resembled those that would happen during NEB in early lactation and feed restriction in mid-lactation cows.

Although the decrease in blood glucose (from 3.6 mmol/L to 3.1 mmol/L) was within the normal reference values (2.55-3.91 mmol/L; Kaneko 1981), by progress in days in milk (DIM) an increasing number of cows (up to 27%; Table 3) had glucose concentrations <2.5 mmol/L, which may be associated with ketosis (Djoković *et al*., 2013). The decrease in blood glucose was about 0.49 mmol/L, higher than the amounts reported for mid-lactation cows. Gross *et al*. (2011) reported that mid-lactation cows with energy-restriction may not become as hypoglycemic as the early-lactation cows. They induced a three-week feed restriction (energy about 50% of the requirements) at about 100 DIM and found 0.16 mmol/L decrease in plasma glucose, much less than 0.65 mmol/L decrease within the first postpartum weeks. In the study of Carlson *et al*. (2006) blood glucose was not affected by energy restriction at about 132 DIM. In a field study (Mohebbi-Fani *et al*., 2012) the level of blood glucose was almost constant from early- to mid-lactation with a non-significant increase in late-lactation. The ration of the cows in the present study contained 28% starch (dry matter (DM) basis) and was potentially glucogenic. It is concluded that, in the absence of a detectable feed restriction under field conditions, mid-lactation cows may experience low levels of blood glucose. This can be due to the high demand for glucose (72 g/kg milk, mostly converted to lactose; Bell, 1995) compared with the effects of interfering factors such as inflammation and stress that may utilize a part of glucose. Continuously elevated levels of haptoglobin and serum amyloid-A (indicators of inflammation and stress) during 21-150 DIM have been reported (Mohebbi-Fani *et al*., 2016). Low blood glucose may be a result of hepatic insufficiency irrespective of the level of energy intake (González *et al*., 1998).

The NEFA concentrations at days 60, 90 and 120 of lactation were 0.35 ± 0.14, 0.41 ± 0.14 and 0.38 ± 0.16 mmol/L, respectively. Cows in 25-80 DIM may have blood NEFA concentrations between 0.12 and 0.38 mmol/L (Bertoni and Trevisi, 2013). In prefresh cows the NEFA values >0.40 mmol/L reveal shortage of energy metabolism. The alarm level is >10% of the population above this threshold (Oetzel, 2004). In the present study, an unexpected finding was that up to 62% of cows (Table 3) showed NEFA concentrations >0.40 mmol/L. In the study of González *et al*. (2011) only 7% of mid-lactation cows (one out of 14) had NEFA concentrations >0.40 mmol/L. Feed restricted cows after 100 DIM had elevated plasma NEFA levels up to 0.23 mmol/L (Carlson *et al*., 2006) and 0.27 mmol/L (Gross *et al*., 2011). In a field study the NEFA level of mid-lactation cows was 0.23 mmol/L (Mohebbi-Fani *et al*., 2012). The high levels of NEFA in the present study could be the consequence of continuous decrease in blood glucose leading to the mobilization of body fats. Whether these high levels of NEFA in mid-lactation cows affect liver functions is under study by our research team.

The BHB concentrations (815 to 890 µmol/L) were higher than those reported in feed restricted cows after 100 DIM by Carlson *et al*. (2006) (450 µmol/L) and Gross *et al*. (2011) (600 µmol/L), but close to the levels reported by Mohebbi-Fani *et al*. (2012) in mid-lactation cows (839 µmol/L). The higher BHB concentrations in the present study can be explained by higher levels of plasma NEFA as substrates of ketone body production. Beta-hydroxybutyrate levels >1200 µmol/L in >10% of fresh cows is suggested as the alarm level for sub-clinical ketosis (Oetzel, 2004; Djoković *et al*., 2013). In the present study 11-13.5% of the cows had BHB levels above 1200 mmol/L (Table 3).

Both T4 and T3 decreased through the study, resembling conditions of feed restriction and NEB. These could be a consequence of decrease in blood glucose. Capuco *et al*. (2001) reported an apparent decrease in serum concentrations of thyroid hormones in feed restricted cows during mid-lactation. Blood concentrations of T4 and NEFA have been reported to be informative for estimation of energy balance in high yielding cows during weeks 1 to 10 postpartum (Reist *et al*., 2002). Decreased circulating thyroid hormones have been reported as a necessary part of adaptations in NEB (Blum *et al*., 1983; Pethes *et al*., 1985; Tiiratz, 1997) and have been reported even after BHB and NEFA returned to normal levels (Eppinga *et al*., 1999). The low T4 and T3 concentrations respectively may extend to mid- and late-lactation stages (Mohebbi-Fani *et al*., 2012). Improvement in feed and energy intake has resulted in rapid increase in T3 levels (Kunz *et al*., 1985).

The inverse correlations of glucose with NEFA as well as its positive relationships with T4 and T3 indicate the influence of glucose on the circulating levels of these metabolites. The inverse correlations of glucose and T4 and positive correlation of NEFA with milk production could mean that the cows with high genetic merit for production may have lower glucose during days 60-120 of lactation leading to lower T4 levels and higher fat mobilization. However, the effect of confounding factors resulting in insufficient feed intake or energy metabolism should be considered. Such changes may affect the reproductive performance (the correlations of glucose and NEFA with reproductive indices). Although the relationship between glucose and BHB has been demonstrated (Kauppinen, 1983), the absence of such a relationship in the present study is inconsistent with Mohebbi-Fani *et al*. (2009). The BHB level in the present study was associated with an extended interval of calving to first heat postpartum. The positive correlation of T3 with calving to first heat interval is not explainable. The T3 levels below 1.4 nmol/L may be associated with diminished heat signs (Jorritsma *et al*., 2003). In the present study the lowest T3 level was 1.39 ± 0.35 nmol/L detected at day 120 of lactation. In the study of Mohebbi-Fani *et al*. (2012) the cows that showed normal or hormonally induced heat signs after 100 DIM had T3 levels of 1.63 and 1.78 nmol/L, respectively.

It is concluded that under field conditions mid-lactation dairy cows may undergo some changes similar to those that usually happen during early lactation or feed restriction. Decrease in glucose concentration may be a key change leading to decreased levels of thyroid hormones and moderately high levels of NEFA and BHB. These metabolic and hormonal changes may affect the general health and performance of the apparently healthy cows. Optimizing herd management and nutrition to maintain adequate glucose supply should be considered during the breeding period in continuation of the previous stages to achieve stabilized hormonal and metabolic conditions.

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Conflict of interest

We declare that we have no conflict of interest.

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