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SUMMARY

The objective of the present study was to investigate the relationship between body condition score (BCS) and some metabolic blood parameters in post-partum (60 ± 10 days) non-pregnant dairy cows. The animals stemming from 2 farms in Erzurum province were assigned into two groups according to their BCS as thin cows (group I, n = 6, BCS: 2.29 ± 0.04 units) and normal cows (group II, n = 8, BCS: 2.81 ± 0.05 units) and in parallel, serum biochemical parameters were determined. BCS has significantly differed between the 2 groups and was significantly correlated with AST (aspartate aminotransferase) activity and BUN (blood urea nitrogen) concentration. In cows with medium BCS, the mean glycaemia was significantly depressed compared to the thin animals whereas significant increases in mean BUN and AST activity were observed. In addition, although the mean values have not significantly differed between the 2 groups, it was also recorded that BHBA (β-hydroxy butyric acid), triglyceride and VLDL (very low density lipoproteins) concentrations and the proportions of free fatty acids tended to slightly increased in the group II, suggesting strengthened lipomobilisation whereas the LDL (low density lipoproteins) concentrations and cholesterolemia at a lesser extent tended to decrease. Slight increases in proteinemia and albuminemia noted in the group II were coupled with mild decreases in minerals, indicating low perturbations in the hydro-electrolytic equilibrium. The fT_3 (free triiodothyronine) concentrations remained similar between the 2 groups. These results suggest that the negative energy balance was more pronounced in cows with medium BCS, leading to a higher risk for metabolic disorders and that thyroid hormones appeared to be weakly involved in the regulation of lipid metabolism in the early lactation period.

Keywords: Dairy cows, early lactation period, body condition score, negative energy balance, serum lipid profiles, metabolism disorders.

RÉSUMÉ

Relations entre l'état corporel et quelques paramètres métaboliques sanguins chez les vaches laitières en début d'allaitement

L'objectif de cette étude était d'étudier les relations possibles entre l'état corporel donné par le BCS (Body condition score) et quelques paramètres biochimiques sanguins chez des vaches laitières non gestantes en période de post-partum (60 ± 10 jours). Les animaux issus de 2 fermes distinctes de la province d'Erzurum ont été regroupés en 2 groupes distincts en fonction de leur BCS [vaches maigres (groupe I, n = 6, BCS: 2.29 ± 0.04 unités) et vaches normales (groupe II, n = 8, BCS: 2.81 ± 0.05 unités)] et les paramètres biochimiques sériques ont été déterminés en parallèle. Les états corporels ont significativement différé entre les 2 groupes et ont été significativement corrélés avec l'activité sérique de l'AST et la concentration du BUN (blood urea nitrogen). Chez les vaches présentant un état corporel correct, la glycémie moyenne a été significativement plus faible que chez les vaches maigres alors que des augmentations significatives de la concentration moyenne du BUN et de l'activité sérique de l'AST ont été observées. De plus, bien que les valeurs moyennes n'aient pas été significativement différentes entre les 2 groupes, il est apparu que les concentrations de BHBA (acide β-hydroxy-butyrique), de triglycérides et de VLDL (lipoprotéines de très faible densité) ainsi que les proportions en acides gras libres ont tendu à augmenter dans le groupe II, suggérant un renforcement de la lipomobilisation, alors que les concentrations de LDL (lipoprotéines de basse densité) et de cholestérol (à un moindre degré) ont tendu à diminuer. Les faibles augmentations de la protéinémie et de l'albuminémie obtenues dans le groupe II ont été associées à des concentrations circulantes diminuées en minéraux, révélant l'existence de perturbations de l'équilibre hydro-électrolytique. Les concentrations de fT_3 (triiodothyronine libre) sont restées similaires entre les 2 groupes. Ces résultats suggèrent que le déficit énergétique du postpartum chez la vache laitière est d'autant plus marqué que le BCS est élevé, conduisant à un risque accru de développer des troubles métaboliques et que les hormones thyroïdiennes apparaissent peu impliquées dans la régulation du métabolisme lipidique durant le début de la période d'allaitement.

Mots clés : Vaches laitières, période d'allaitement, état corporel, déficit énergétique, profils lipidiques sériques, troubles métaboliques.

Introduction

High-yielding dairy cows are typically in a state of negative energy balance (NEB) during early lactation period because the amount of energy required for the maintenance of body tissue functions and milk production exceeds that the cows can consume [29]. Mobilisation of body energy reserves during the early lactation enables the cow to close the gap between the alimentary energy intake and its loss through the milk production [38]. Since the alterations in energy reserves have a considerable influence upon the productivity, health and reproduction of dairy cows [29, 43, 45], the monitorisation of optimal management of energy reserves is obviously needed. For farmers and veterinarians, it is therefore important to have an efficient and practically applicable tool to estimate the body reserves in dairy cows [6]. In this context, the monitorisation of changes in those reserves using body weight (BW) measurements is one of the methods used routinely. However, the BW changes are influenced by factors other than the fluctuations of the body fat amount, including changes in the endogenous protein and water contents, gastrointestinal content, changing organ weights, foetal development and frame size [38]. Instead, presumably, the metabolic and hormonal factors might be used to assess the energy balance in a properly-timed manner rather than using indirect measures of energy reserves, which are always retrospective. Although those measures would provide a more objective assessment of energy balance, each/any measurement has its own limitations. Those techniques have disadvantages, requiring the blood sampling and cost of analysis equipment. Therefore, the primary method to be used in dairy industry is a subjective measurement of the amount of body condition, termed as the BCS. This technique is accomplished by the visual or tactile observation, or both, of a cow by trained personnel [2].

The BCS has been widely accepted as the most practical method for assessing the changes in energy reserves in many species, including dairy cattle [2]. It is a subjective estimate of the metabolisable energy reserves in the adipose tissue. Its evaluation is based on the outer appearance of cow that interacts with its body fat reserves and, therefore, is directly influenced by the energy balance [38]. LOWMAN *et al*. [23] were the first to introduce a BCS scale (4-point) for dairy cows, adapting a scoring system used to rank the beef cattle. Further, the scale used to measure the BCS (5-, 6-, 8-, and 10-point scales) differs between the countries [13, 24, 34]. But, collectively, the low values always reflect the emaciation while the high values equate to obesity [33].

TREACHER *et al.* [42] demonstrated that the fat cows experienced markedly more cases of periparturient disorders than the thin cows. However, in the literature, the association between the absolute BCS or the changes in BCS and health disorders was quite variable [14]. So far, a number of periodic studies were conducted upon the monitorisation of BCS and its relationship with the metabolic profiles of cows at the same periparturient period [1, 4, 5, 22, 30, 36]. To our knowledge, however, no such study could be found in the literature upon the relationship between different BCS scores and the metabolic or hormonal parameters in cattle. In the present study, therefore, the metabolic parameters of dairy cows with different BCS scores were investigated during the early lactation period.

Material and Methods

ANIMALS AND PROTOCOL DESIGN

In this study, 14 periparturient (60 ± 10 days postpartum, as the end of early lactation period) multiparous dairy Holstein cows, 4 to 10 years old, were used. The animals, proved to be non-pregnant by rectal palpation, were stemming from the two different regional farms in Erzurum province, Turkey.

Body conditions were scored as fairly low (Group I: < 2.50 units; n = 6) and medium categories (Group II: > 2.50 units; n = 8) using a scale from 1 (emaciated status) to 5 (extremely fat) [13], mainly assessed with the quarterly increments by the same well-experienced person. Clinical inspection (respiration rate, pulsation, temperature, and rumen motility as well as the reproductive health), total milk productions, diet contents of all animals, BCS determination and blood sampling were conducted on the same day.

All cows from the group I were fed daily with 10 kg grass hay, 3 kg mixed silage (corn, clover, trefoil, vetch), and 2 kg concentrate for milking and were stemming from the same farm while those from the group II daily received 1.9 kg grass hay, 3.7 kg clover, 9 kg corn silage, 8.4 kg residual sugar beet silage, and 7 kg concentrate for milking and were belonging to the second farm. Drinking water was provided *ad libitum* in easily accessible containers made either of stainless metal or of plastic material. All the experimental animals were housed freely in the indoor shelters with sufficient bedding and air conditioning together with semi-free access to the outdoor pens especially during the day time.

Blood samples (10 mL) were collected by puncture of V. jugularis into sterile microtubes (BD Vacutainer System, Plymouth, UK). After clotting at room temperature for 30 minutes and centrifugation (3000 g for 15 minutes at 4°C) sera were carefully harvested and stored at -20°C until analysis. All samples were monitored at the same time.

BIOCHEMICAL ANALYSES

The concentrations of serum glucose, triglyceride, cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL), total proteins, albumin, blood urea nitrogen (BUN), creatinine, calcium (Ca), phosphorus (P), chloride (Cl), sodium (Na), potassium (K) and the serum activity of aspartate aminotransferase (AST) were determined using an automatic biochemical analyzer according to the manufacturer guides (Cobas 6000 analyzer, Roche). However, serum free triiodothyronine (fT_3) was determined separately using a chemiluminescence Immunoassay (CLIA) test (E-170 Analyzer, Roche). The concentrations of very low density lipoproteins (VLDL) were calculated by dividing the triglyceride concentrations by a factor 5. The concentrations of blood β-hydroxybutyric acid (BHBA) were determined using the β ketone test strip (Optimum β ketone Test Strips, Lot No: 7599, Abbott Diabetes Care, Abingdon, UK) [21].

For total lipid extraction, sera (500 μL) were vigorously mixed with 500 μL n-hexane/iso-propanol (2:1 (v/v)) and centrifuged (2000 g, 15 minutes, 4°C). The upper phase was used for high performance thin layer chromatography (HPTLC) analysis [19]: extracted lipids (5 μL) were spotted with a micropipette 2 cm above the bottom of HPTLC plates and they were developed 6 cm from the application point using a mobile phase of hexane: diethylether: formic acid (80:20:2 (v/v/v)). To visualise lipid classes, the entire plate was sprayed with a 10% $CuSO_4$ (w/v), in 8% H_3PO_4 (v/v) and charred at 180°C. After cooling, HPTLC plates were evaluated by Phoretix 1D (TL120) software [40].

STATISTICAL ANALYSIS

Serum concentrations of glucose, BHBA, triglyceride, cholesterol, LDL, HDL, VLDL, total proteins, albumin, BUN, creatinine, Ca, P, Cl, Na, K, AST and fT_3 of both BCS groups (as fairly low or medium scores) were analysed by one-way analysis of variance (ANOVA) using MINITAB statistical software programme (Release 11.2; MINITAB Inc., Pennsylvania, USA). The positive or negative correlations between all the blood parameters concerned and BCS were analysed by using Pearson's correlation coefficients. The values were represented as the mean ± standard error of the mean (SEM). Differences between the mean values of serum metabolites or hormone concentrations according to the BCS groups were considered significant when $P < 0.05$.

Results

Upon the clinical examination, the respiration rate, pulsation, temperature and rumen motility were within the usual limits in cows from both groups. Furthermore, the reproductive health of experimental cows was proved to be normal by inspection and rectal palpation with no apparent vaginal discharge, uterine infection or ovarian disorders (cysts, tumours, etc.). The BCS was significantly lower in the group I regrouping the thinner animals (2.29 ± 0.04 units; extreme values: 2.10 - 2.35) than in the group II (2.81 ± 0.05 units; extreme values: 2.65 - 3.00) ($P < 0.001$). Furthermore, on the day of body condition scoring, the average milk daily productions were 13.1 L and 15.2 L in groups I and II, respectively.

Serum biochemical values according to the BCS were summarised in Table I. As it was noted, the overall serum lipid profile (triglyceride, cholesterol, ketone bodies (BHBA) and lipoprotein concentrations) has not significantly differed according to the BCS in cows in early lactation period; however, the serum LDL concentrations tended to be lowered in cows exhibiting a medium BCS (group II) compared to the thinner cows (group I) ($P = 0.061$). The proteinemia tended to be depressed in thin cows (group I) compared to the others (group II) ($P = 0.078$) but differences in total protein and in albumin concentrations between the 2 groups were not significant. By contrast, the mean serum glucose concentration was significantly higher in the group I than in the group II ($P < 0.05$). Additionally, although the mean creatinine concentrations did not significantly differ between the 2 groups, the BUN values were dramatically higher ($P < 0.001$) in the group II than in the group I. In parallel, a strong increase in the serum AST activity was also recorded in cows with medium BSC ($P < 0.01$). Considering the serum Ca, P, Cl, Na and K concentrations in one hand and the fT_3 concentrations in the other hand, there were no significant differences between the two BCS groups. In one hand, the BCS was significantly and negatively correlated with LDL concentrations only but there was a negative tendency for glycaemia, cholesterolemia, chloremia and kaliema. On the other hand, significant positive correlations were obtained with BUN, creatinine concentrations and the AST activity, but there was a positive tendency for proteinemia, albuminemia, BHBA and fT_3 concentrations (Table II).

The overall data from the total lipid profiles [triglyceride, cholesterol, non-esterified or free fatty acids (NEFA), polar lipid (PL) hydrocarbon (HC) proportions] of cows from groups I and II are given collectively in Table III. Although

Biochemical parameters	**Group I (n = 6) (BCS < 2.50 units)**	**Group II (n = 8) (BCS > 2.50 units)**	**Significance**
Glucose (mmol/L)	2.91 ± 0.04	2.31 ± 0.26	*P < 0.05*
BHBA (mmol/L)	0.48 ± 0.07	0.83 ± 0.23	NS
Cholesterol (mmol/L)	3.60 ± 0.37	3.22 ± 0.25	NS
VLDL (mg/L)	35.0 ± 2.5	37.0 ± 3.7	NS
LDL (mg/L)	236.7 ± 110.4	145.0 ± 53.2	NS
HDL (mg/L)	998.3 ± 99.3	988.7 ± 71.9	NS
Triglycerides (mg/L)	175.0 ± 12.6	185.0 ± 18.3	NS
Total proteins (g/L)	72.4 ± 4.3	82.6 ± 3.2	NS
Albumin (g/L)	28.7 ± 1.6	31.8 ± 1.4	NS
BUN (mg/L)	21.2 ± 2.4	101.0 ± 5.8	*P < 0.001*
Creatinine (μmol/L)	75.22 ± 5.31	83.19 ± 3.54	NS
AST (U/L)	63.50 ± 5.04	91.00 ± 5.54	*P < 0.01*
Ca (mmol/L)	2.30 ± 0.03	2.29 ± 0.05	NS
P (mmol/L)	1.45 ± 0.09	1.34 ± 0.13	NS
Cl (mmol/L)	93.67 ± 1.23	91.00 ± 1.00	NS
Na (mmol/L)	129.83 ± 0.48	129.38 ± 1.24	NS
K (mmol/L)	26.18 ± 0.45	20.91 ± 2.76	NS
fT_3 (pmol/L)	3.35 ± 0.18	3.43 ± 0.23	NS

BCS: Body condition score; BHBA: β-hydroxybutyric Acid; VLDL: very low density lipoproteins; LDL: low density lipoproteins; HDL: high density lipoproteins; BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; Ca: Calcium; P: Phosphorus; Cl: Chloride; Na: Sodium; K: Potassium; fT_3: free triiodothyronine; NS: not significant.

TABLE I: Serum biochemical parameters according to the BCS categories of dairy cows in early lactation period. Results are expressed as mean ± standard error of the mean (SEM).

Biochemical parameters	Correlation coefficient	Significance
Glucose	r = -0.341	NS
BHBA	r = 0.265	NS
Cholesterol	r = -0.348	NS
VLDL	r = 0.096	NS
LDL	**r = -0.514**	***P < 0.05***
HDL	r = -0.166	NS
Triglycerides	r = 0.096	NS
Total proteins	r = 0.403	NS
Albumin	r = 0.338	NS
BUN	**r = 0.888**	***P < 0.001***
Creatinine	**r = 0.529**	***P < 0.05***
AST	**r = 0.673**	***P < 0.01***
Ca	r = -0.104	NS
P	r = -0.169	NS
Cl	r = -0.471	NS
Na	r = -0.114	NS
K	r = -0.290	NS
fT_3	r = 0.269	NS

BCS: Body condition score; BHBA: β-hydroxybutyric Acid; VLDL: very low density lipoproteins; LDL: low density lipoproteins; HDL: high density lipoproteins; BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; Ca: Calcium; P: Phosphorus; Cl: Chloride; Na: Sodium; K: Potassium; fT_3: free triiodothyronine; NS: not significant.

TABLE II: Correlations between BCS and serum biochemical parameters in dairy cows in early lactation period. Significant correlations were expressed in bold.

the proportions of free fatty acids appeared to be slightly increased in cows with medium BCS, no significant difference in lipid proportions was evidenced according to the body conformation.

Discussion

Variations in the BCS in lactating dairy cows are reflected by the changes of subcutaneous fat thickness, diameter of *M. longissimus dorsi* as well as the metabolic and endocrine statues [1, 5, 30]. Both the literature and field practices have shown that body condition influences productivity, reproduction, health, and survival in cattle [29, 43, 45]. The greatest benefits from managing the cows for optimal BCS at each stage of the lactation likely come from the advancements in animal health [44]. The fat or thin state of cows can constitute an indicator of nutritional, metabolic disorders, health problems or can indicate poor management at farm level [45]. In the present study, relationships between the BCS and some metabolic blood parameters in periparturient dairy cows (60 ± 10 days post partum) were investigated.

Body fat has a negative feedback effect upon the dry matter intake (DMI), increasing fatness causing DMI reduction [16]. Therefore, DMI is generally greater in cows having a low condition [42]. In dairy cows, increased energy demands observed with the progress of lactation result in a decrease in glycaemia and an increase in the concentrations of free fatty acids and ketone bodies [8]. In this respect, CAVESTANY *et al*. [7] noted that this metabolic status might be related to

Lipid	Group I (n = 6) (BCS < 2.50 units)	Group II (n = 8) (BCS > 2.50 units)	Significance
Triglyceride (%)	5.16 ± 0.54	4.71 ± 0.78	NS
Cholesterol (%)	17.70 ± 0.30	17.51 ± 0.27	NS
NEFA (%)	2.94 ± 0.42	3.62 ± 0.78	NS
PL (%)	24.06 ± 1.06	24.87 ± 0.90	NS
HC (%)	50.15 ± 1.66	49.29 ± 1.02	NS

NEFA: non-esterified fatty acids; PL: polar lipids; HC: Hydrocarbon; NS: not significant.

TABLE III: Serum lipid profiles expressed in % of total lipids according to the BCS categories of dairy cows in early lactation period. Results are expressed as mean ± standard error of the mean (SEM).

insufficient composition of the ration or low dry matter intake. Indeed, fat deposited in adipose tissue as triglycerides were mobilised as free fatty acids (FFAs) after hydrolysis of glycerol ester bonds in case of high energy demand such as in lactation [33]. The released FFAs (or non esterified fatty acids) offer 3 metabolic interests: firstly, they are utilised for milk fat synthesis by the mammary gland, secondly they also constitute an energy source for peripheral tissues and finally they are again esterified into triglycerides by the liver [11] and exported throughout VLDL formation [33]. Complete oxidation of FFAs generates metabolites (acetyl coenzyme A) that are degraded via the Krebs cycle to generate energy. However, if the Krebs cycle gets overloaded, the acetyl Co A residues are shunted off to produce ketone bodies such as β ketobutyrate, acetone and β hydroxybutyrate or BHBA [10]. Consequently, in thin cows with an insufficient lipomobilisation capacity (BCS ≤ 2.50 units), the FFA, triglyceride and VLDL concentrations may be low as reported by YAYLAK *et al*. [46]. In the present study, cows with medium BCS exhibited significantly lower serum glucose concentrations than the thinner cows; increased NEFA proportions, and increased serum VLDL, triglyceride and BHBA concentrations were also recorded in this group although the differences in mean values were not statistically significant between the 2 distinct BCS categories. Although HEUER *et al*. [20] and REIST *et al*. [29] could not find a marked relationship between BCS and ketosis, the most consistent relationship among the published studies has been the increased incidence of ketosis in cows with high BCS at dry-off and calving periods [12, 14, 17, 28]. The normal circulating concentration of ketone bodies (using BHBA as an indicator) is less than 100 mg/L, i.e. 0.96 mmol/L [3]. Clinical ketosis is defined as a concentration of ketone bodies higher than 350 mg/L (3.37 mmol/L) and concentrations comprised between the two limits are referred as subclinical ketosis [10]. In this regard, 3 cows belonging to the second group have exhibited intermediate BHBA concentrations (comprised between 0.96 and 3.37 mmol/L) and could be considered as developing subclinical ketosis.

The relationship between the cholesterolemia and energy balance has not yet been completely defined. Some authors [4-7] suggested that changes in the cholesterol metabolism during the periparturient period were not associated with the BCS status. However, RUEGG *et al.* [37] reported that the cholesterol concentrations during the early lactation period were inversely related to the loss of body condition; in agreement with that, cholesterolemia was slightly, but not significantly, higher in thin cows than in cows with a medium BCS. On the other hand, the variations in cholesterol concentrations may be also partly explained by a low DMI compromising the exogenous cholesterol supply [18]. Corroborating that, in the present study, the circulating LDL concentrations, mainly transferring cholesterol towards peripheral cells, tended to increase, but not significantly, in the thinner cows (group I) compared to the others ($P = 0.061$) whereas the HDL concentrations, primarily transferring cholesterol from peripheral cells to hepatocytes, were similar in the 2 groups.

During the early lactation, since the increase in milk production is greater than the DMI [39], the early lactation *per se* may probably cause ionic depletion. In this study, it should be emphasised that there was no marked change in any of the minerals studied, i.e. Ca, P, Cl, Na and K, even if they tended to be lowered (particularly chloremia and kaliemia, $P = 0.115$ and $P = 0.142$, respectively) in the group II. Considering individual values, hypocalcaemia (< 8 mg/L, i.e. 2.00 mmol/L) was recorded in one cow from the group II. In addition, proteinemia and albuminemia at a lesser extent tended to be slightly increased in cows with medium BCS ($P = 0.078$ and $P = 0.180$, respectively), suggesting a relative dehydration. However, the alterations in the hydro-electrolytic equilibrium, induced by the considerable amount of daily milk production (approximately 15 L for around 60 days), have remained moderate, probably because of the satisfactory amounts of post-calving DMI in both groups.

As the increased milk production in early lactation was further supported by the catabolism of tissue reserves comprising endogenous proteins [35], the marked increase in the serum BUN concentrations ($P < 0.001$) and in the serum AST activity ($P < 0.01$) observed in cows with medium BCS may be related to a dramatically increased catabolism of endogenous proteins, particular from muscles, which may constitute a prerequisite for milk protein synthesis and excretion during lactation [25]. Indeed, the average duration of negative energy balance (NEB) in dairy cows endures about 8 weeks [9], ranging from 5 weeks [41] to 14 weeks [15]. It was previously reported that plasma concentrations of glucose, cholesterol, BUN and fT_3 positively correlated with the energy balance whereas those of BHBA, NEFA, creatinine, albumin, and enzyme activities such as AST, LDH and GLDH negatively correlated [22, 29]. In the present study, it was found that the BCS in periparturient cows was significantly and negatively associated with LDL concentrations only but a negative tendency was also evidenced with glycaemia, cholesterolemia, chloremia and kaliemia whereas significant positive correlations were obtained with BUN and creatinine concentrations as well as the serum AST activity but a positive tendency for proteinemia, albuminemia, BHBA, and fT_3 concentrations was also noted. Consequently, it was suggested that the negative energy balance was more severe in cows with medium or elevated BCS than in thin cows for a roughly similar milk production. Surprisingly, the serum fT_3 concentrations were similar between the 2 groups of periparturient cows, although the energy balance was more deficient in the group II than in the group I and consequently serum fT_3 concentrations appeared to be relatively independent from the BCS in the present experiment. This result suggests that in the early lactation period, the active thyroid hormone was not directly involved in the metabolism regulation in cows.

Apart from the effects of genetic selection upon the BCS [31], several other factors at the animal level have been reported to affect BCS in cows, including the parity [32], age within parity [27] and calving season [26]. Moreover, the management factors (at the herd level) such as population [32], amount of feeding and type of diet [31] have also been reported to affect the morphological parameter. Herein, although the animals in both groups were virtually at the same lactation stage (near the end of the early lactation period), with no marked difference in their daily milk production (less than 2 L), marked differences in BCS were noted, probably related to variations in the feeding amount and diet type during the earlier dry period and subsequent calving time.

As a conclusion, the present study showed that the risk for development of metabolic disorders such as hypoglycaemia, ketosis and hypocalcaemia in the postpartum period (at the end of early lactation) was higher in cows with medium BCS (2.81 ± 0.05 units) than in thin animals (2.29 ± 0.04 units).

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