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CHANGES IN LIPID AND MINERAL PROFILES IN COWS WITH SUBCLINICAL AND CLINICAL KETOSIS

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The aim of the present study was to establish the changes in lipid and mineral metabolism in cows from the Holstein breed with ketosis. Blood samples were obtained from 158 cows for determination of β-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), total cholesterol (TC), high-density lipoproteins (HDL-C), low-density lipoproteins (LDL-C), triglycerides (TG), calcium (Ca), phosphorus (P) and magnesium (Mg). The cows were divided in three groups: pregnant, recently calved and lactating. Target cows were classified as healthy (C), affected with subclinical (SCK) and with clinical ketosis (CK) depending on their blood BHBA levels.

The levels of NEFA in cows with SCK were elevated, while in cows with CK – decreased. The quantities of total cholesterol and HDL-C decreased, while the blood levels of LDL-C and TG were increased in cows with SCK and CK ketosis. The parameters of the mineral profile Ca, P and Mg were decreased in cows with SCK and CK.

Key words: ketosis, cholesterol, mineral profile, cows.

Introduction

Ketosis is a metabolic disorder that occurs in high-producing dairy cows during the transition period when energy requirements exceed energy intake. This period is characterized by a decrease in dry matter intake (by over 30%), the development of a negative energy balance (NEB) and metabolic changes that can lead to an increased incidence of ketosis and reduced animal productivity (Samiei et al. 2015). From a clinical point of view, ketosis can be classified as subclinical or clinical, depending on the amount of ketone bodies in the organism and the presence of clinical symptoms (Gordon et al. 2013). Based on various reports, the incidence of SCK can range from 15 to 53 % and CK from 1.6 to 23% (Brunner et al. 2019). Approximately 50% of dairy cows are affected by SCK in the first month of lactation (Loiklung et al. 2022). Economic losses from the disease are expressed in reduced milk yield and body weight, low insemination rate and increased rates of diseases such as metritis, mastitis and abomasal displacement (Cainzos et al. 2022).

Blood BHBA concentrations reflect the degree of NEB and lipid mobilization in dairy cows, therefore they are diagnostic markers for SCK and CK (Sordillo et Raphael 2013). Different blood levels of BHBA have been reported in cows with SCK – from 1.0 mmol/l to 2.9 mmol/l (Macrae et al. 2019), while in cows with CK - from 2.0 mmol/l to 3.0 mmol/l (González et al. 2011). The second early marker for the degree of fat mobilization from fat depots is NEFA (Choi et al. 2023). Non-esterified fatty acids are the main component of triglycerides. Under the influence of hormone-dependent lipase, TG are hydrolyzed, which leads to the release of NEFA and glycerol (Allen et Piantoni 2013). Non-esterified fatty acids can be directly used as a fuel source by the tissues, for the synthesis of milk fat or absorbed by the liver (Mann 2022). Its blood levels are affected by various factors related to feeding time, ration, sampling methodology and time of sample collection and

storage, animal stress during blood sampling, and anticoagulants used (Leroy et al. 2011). According to Hiss et al. (2009), NEFA values <0.3 mEq/l should be considered as normal in pregnant cows, and <1.0 mEq/l on the day of calving. After the third day of lactation, values should be <0.7 mEq/l. Nogalski et al. (2012) reported that cows with values of BHBA – 0.52 mmol/l and NEFA – 0.29 mmol/l before parturition developed ketosis in the postpartum period.

Macrae et al. (2019) found that blood TG and cholesterol levels can be used as indicators of energy status in ketotic cows, in parallel with NEFA and BHBA. Choi et al. (2023) found an increase in TG, NEFA and hypoglycemia in cows with SCK and CK, while González et al. (2011) and Djoković et al. (2013) reported low TG levels (<0.1 mmol/l) in cows with SCK during the first weeks after parturition, finding no correlation between blood TG and NEFA levels. Grum et al. (1996) reported unreliable changes in cholesterol levels in cases of hyperketonemia, whereas Basoglu et al. (1998) found that during the transition period, NEB can lead to high serum cholesterol values in cows in ketosis. According to Bobe et al. (2004) intensive lipomobilization, associated with accelerated ketogenesis and lipogenesis in the liver, leads to lower levels of glucose, TG, total cholesterol and HDL-C in cows with ketosis without changes in LDL-C values.

Minerals are necessary elements for every organism as structural components of a number of enzymes and to support growth, reproduction, lactation, etc. (Oetzel 2013; Del Valle et al. 2015). Calcium is an element involved in maintaining muscle tone and motility of the gastrointestinal tract. Its low concentrations in ketosis and other metabolic diseases lead to a decrease in food intake (as a result of decreased rumen motility), an increase in the incidence of abomasal displacement and a decrease in milk yield (Oetzel 2013; Choi et al. 2023).

Phosphorus metabolism is directly related to carbohydrate metabolism. Phosphorus included in the composition of phospholipids, and together with calcium, in the composition of teeth and bones (Krajnicakova et al. 2003). Hypophosphatemia often observed in early lactation because milk production requires significant amount of phosphorus and colostrum has a higher phosphorus content than milk. Therefore, the greatest challenges to phosphorus homeostasis occur in early lactation (Grünberg 2014). Sahinduran et al. (2010) found decreased blood levels of Ca and P in ketotic cows as a result of decreased calcium intake due to suppressed appetite and decreased absorption; as a result of hyperketonemia or other diseases. Fatur (1997) reported high levels of P in dairy cows with ketosis. Bigner et al. (1996) found that cows fed calcium-poor rations prior to calving were more insulin resistant and more prone to ketosis. DeGaris et al. (2010) found a positive relationship between body condition score and Ca levels after calving, as well as a positive correlation between Ca and blood glucose values, before and after calving.

Magnesium is required for normal skeletal development and is one of the main activators of the enzymes involved in the Krebs cycle (Tanritanir et al. 2009). Kaya et al. (2016) reported low magnesium and phosphorus levels in cows with clinical ketosis, and Simeonov (1978) found no changes in sodium, potassium and magnesium levels in ketosis.

The aim of this study was to investigate high-yielding cows in different physiological conditions (pregnant, recently calved and lactating) with diagnosed SCK and CK to detect changes in lipid and mineral profiles.

Materials and methods

Animals

A total of 158 cows from the Holstein breed in 1st and 4th lactation with average weight 450-550 kg were included in the study.

Experimental design

The cows were divided into three groups according to their physiological condition: I group – pregnant cows (from day 15 to day 0 pre-calving); II group – recently calved (from day 0 to 15 postpartum) and III group – lactating (from day 30 to 45 postpartum). Blood chemical analysis of BHBA concentrations was performed in all cows in order to classify them as control (C, BHBA <1.2 mmol/l), affected with SCK (BHBA from 1.2 to 2.6 mmol/l) and CK (BHBA >2.6 mmol/l). The different groups of cows were reared under equal conditions and fed according to their physiological status.

Group I included 21 pregnant animals – 9 healthy (C) and 12 (57%, with SCK). Blood BHBA levels indicative for CK were not established in this group. Group II (n=90, recently calved) – 55 (C), 27 (30%, with SCK) and 8 (8.88%, with CK) and group III (n=47, lactating cows) – 24 (C), 15 (32%, with SCK) and 8 (17%, with CK).

Blood samples and analyses

Blood samples were collected through puncture of the coccygeal vein using sterile 21G needles and vacutainers without anticoagulant and with heparin (5 ml, Biomed, Bulgaria). Samples were obtained in the morning before feeding and were stored and transported at 4^0C. Analysis was performed within 24 hours after sampling. Blood BHBA levels were determined in situ using a portable Xpress-I system (Nova Biomedical, UK). The following indices were determined: non-esterified fatty acids (NEFA, mmol/l), total cholesterol (TC, mmol/l), high-density lipoproteins (HDL-C, mmol/l), low-density lipoproteins (LDL-C, mmol/l), triglycerides (TG, mmol/l), calcium (Ca, mmol/l), phosphorus (P, mmol/l) and magnesium (Mg, mmol/l). Biochemical investigations were analyze on an automated biochemical analyser Mindray BS-120 (China) and Integra 400 plus Roch (F. Hoffmann – La Roche Ltd., Switzerland). The values of NEFA in the blood serum determination using NEFA ELISA Kit (Changhay Crystal Day Biotech Co., LTD., China) and ELISA Reader Sunrise (Tecan, Switzerland).

Statistical analysis

Statistical analysis was done with Statistica 6.0, StatSoft, Inc. (USA, 1993) and ANOVA test. Results were presented as mean (x) ± standard deviation (SD). The level of statistically significance was $p<0.05$.

Results

The results from the biochemical analysis in the three groups of cows are shown in Table 1.

Blood BHBA concentrations in control cows from the three groups were within the reference range. In cows with SCK they were statistically significantly increased as vs controls (Table 1). The blood analysis in this parameter in cows with CK from groups II (recently calved) and III (lactating), showed that BHBA levels were statistically significantly higher ($p<0.001$) than respective C and SCK groups (Table 1). In pregnant cows from group I concentrations of BHBA higher than 2.6 mmol/L were not exhibited, e.g. CK was not present.

Table 1: Changes in blood BHBA and NEFA levels in Holstein cows from I, II and III groups with SCK and CK (mean ± SD).

		BHBA (mmol/l)	NEFA (mmol/l)
Group I (15–0 days pre-partum)	Control	0.52±0.09	0.31±0.02
	Subclinical ketosis	1.65±0.63[c]	0.48±0.03[a]
Group II (0–15 days postpartum)	Control	0.43±0.25	0.76±0.05
	Subclinical ketosis	1.73±0.61[c]	0.84±0.03[a]
	Clinical ketosis	4.27±1.29[c]	0.68±0.02
Group III (30–45 days postpartum)	Control	0.30±0.16	0.42±0.01
	Subclinical ketosis	1.57±0.55[a]	0.56±0.04[a]
	Clinical ketosis	4.75±1.36[c]	0.35±0.01

Legend: [a]p<0.05; [b]p<0.01; [c]p<0.001; 1-vs control groups

Blood serum NEFA values of the three control groups ranged within the reference interval (Table 1). In the groups I, II and III with SCK, blood NEFA concentrations were statistically significantly higher than controls (p<0.05). All cows with CK (group II and III) exhibited lower blood NEFA levels vs controls (Table 1).

The results of the blood analysis of the lipid profile of the cows of the first, second and third groups with SCK and CK are shown in Table 2.

Table 2: Changes in lipid profile in Holstein cows from I, II and III groups with SCK and CK (mean ± SD).

Parameters	**I group (15-0 days pre-partum)**		**II group (0-15 days postpartum)**			**III group (30-45 days postpartum)**		
Groups	**C**	**SCK**	**C**	**SCK**	**CK**	**C**	**SCK**	**CK**
Total cholesterol (TC) (mmol/l)	3.33±0.5	1.68±0.2[1a]	3.63±0.1	2.00±0.6[1a]	1.82±0.5[1a]	3.76±0.1	2.39±0.3[1a]	2.19±0.5[1b]
High-density lipoproteins (HDL-C) (mmol/l)	1.2±0.02	0.60±0.01[1b]	1.35±0.01	0.8±0.01[1a]	0.32±0.02[1c]	1.07±0.01	0.75±0.02[1a]	0.49±0.03[1c]
Low-density lipoproteins (LDL-C) (mmol/l)	3.2±0.3	3.90±0.2	3.28±0.6	4.24±0.2[1b]	4.08±0.1[1a]	3.65±0.1	4.43±0.6[1a]	4.38±0.4[1a]
Triglycerides (TG) (mmol/l)	0.12±0.04	0.23±0.03	0.10±0.03	0.23±0.04[1a]	0.34±0.01[1b]	0.14±0.01	0.22±0.02	0.34±0.01[1a]

Legend: [a]p<0.05; [b]p<0.01; [c]p<0.001; 1-vs control groups

Blood total cholesterol, HDL-C, LDL-C and TG levels in the three control groups of cows were within the physiological range (Table 2). After examination of the levels of TC and HDL-C of the cows of the three groups with SCK, it was found that its values decreased significantly compared to those of the control groups (p<0.05; p<0.01), while the values of LDL-C and TG increased vs controls (p<0.05; p<0.01) (Table 2). The changes in the lipid profile in the blood serum of the cows from the two groups with a CK changed in the same directions as in the cows with SCK, namely: TC and HDL-C were decreased (hypocholesterolemia) (p<0.05; p<0.001), while LDL-C and TG were increased, compared to the control groups (p<0.05; p<0.01) (Table 2).

The changes in the levels of Ca, P and Mg in the blood of cows from the control groups are presented in Table 3, which shows that its values vary around the physiological norms for this animal species. The examination of the levels of Ca, P and Mg in the blood serum of cows of the three groups with signs of SCK shows that its values decreased compared to control groups ($p<0.05$; $p<0.01$). The changes in the amounts of Ca, P and Mg in the serum of cows of the two groups (recently calved and lactating) with CK change in the direction of a significant decreased compared to the control groups ($p<0.05$; $p<0.01$) (Table 3).

Table 3: Changes in mineral profile in Holstein cows from I, II and III groups with SCK and CK (mean ± SD).

Parameters	I group (15-0 days pre-partum)		II group (0-15 days postpartum)			III group (30-45 days postpartum)		
Groups	C	SCK	C	SCK	CK	C	SCK	CK
Calcium (Ca) (mmol/l)	2.86±0.2	2.32±0.1	2.89±0.2	2.02±0.2	1.81±0.4^{1b}	2.95±0.2	1.84±0.2^{1b}	1.74±0.2^{1b}
Phosphorus (P) (mmol/l)	1.94±0.1	1.06±0.2	1.95±0.3	1.48±0.3	1.27±0.2^{1a}	1.86±0.4	1.22±0.3	0.96±0.4^{1b}
Magnesim (Mg) (mmol/l)	0.94±0.1	0.87±0.2	0.81±0.1	0.63±0.1	0.51±0.2^{1b}	0.85±0.3	0.64±0.2	0.55±0.2^{1b}

Legend: $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$; 1-vs control groups

Discussion

The transition period is a critical stage for a dairy cows. Its characterized by drastic metabolic changes, that occurs as a result of increased energy needs, accompanied by insufficient or inadequate nutrition (Moghaddam et Hassanpour 2008). The decreased blood glucose level leads to increased lipomobilization and stimulation of ketogenesis for maintenance of metabolic homeostasis. Increased concentrations of ketone bodies in blood during hyperketonemia are a response to occurring carbohydrate deficiency and tricarboxylic acid cycle inhibition (Reece, 2004).

In our research we found that the cows suffered from SCK and CK during the late pregnancy, calving and lactation. Blood BHBA levels reached 1.73 mmol/l in animals with SCK and 4.75 mmol/l in cows with CK. High blood BHBA values reveal the incomplete oxidation of NEFA in the Krebs cycle during NEB (Doepel et al. 2002).

This research demonstrated that blood levels of NEFA as an indicator of negative energy balance change in different directions: in dairy cows with SCK from the three groups (pregnant, recently calving, lactating) NEFA levels were increased vs controls. This results support the hypothesis that enhanced lipolysis in lactation leads to the formation of large amounts of NEFA that can not be metabolized in the liver (Roche et al. 2013). In the three groups of cows with CK, NEFA levels decreased. Approximately 15 to 20% of NEFA circulating in the blood is processed in hepatocytes by esterification (Roche et al. 2013). The resulting esterified fatty acids have several alternative pathways. The first is to be used directly for energy by the liver through oxidation. The second is to bind to glycerol and become TG, which are either packaged (with cholesterol, cholesterol esters, phospholipids and proteins) and exported as very low-density lipoproteins (VLDL) to adipose tissue, or stored as triacylglycerol (TAG) in hepatocytes (hepatocellular lipidosis) (Roche et al. 2013). The

third possible way is by entering the mitochondria (carnitine dependent process) to undergo β-oxidation to Acetyl CoA (Roche et al. 2013). Acetyl CoA combines with oxaloacetate in the Krebs cycle (to citrate), producing energy. In cases where oxaloacetate is in insufficient quantity, i.e. it is used as a substrate for gluconeogenesis, Acetyl CoA is used to form ketone bodies. During the complete oxidation of NEFA, Acetyl CoA stimulates the Krebs cycle and provides energy for gluconeogenesis from pyruvate (Ingvartsen 2006). In addition to unlocking gluconeogenesis, TG, NEFA, and ketone bodies can act together as alternative fuel sources for many tissues in the body (Sordillo et Raphael 2013).

From the blood serum analysis of the cows from the three groups with different physiological conditions, we found that the parameters of the lipid profile TC, HDL-C, LDL-C and TG change in different directions. The concentrations of total cholesterol and HDL-C decrease and reach below 0.5 mmol/l in cows with SCK and CK, whiele the levels of LDL-C and TG increased and reached above 4 mmol/l in cows with both forms of ketosis. Similar results were obtained by Bertoni et Trevisi (2013), finding an increase in TG and NEFA, hypoglycemia and a decrease in total cholesterol in cows with SCK and CK. Grum et al. (1996) reported an increase in TG and no significant changes in cholesterol levels in cows with SCK, with TAG concentrations generally increasing in parallel with total lipids. Djoković et al. (2013) reported low TG levels in animals with SCK.

Intensive lipomobilization, associated with accelerated ketogenesis and lipogenesis in the liver, leads to a decrease in the levels of glucose and total cholesterol in the blood of cows with fatty liver dystrophy and ketosis. A negative correlation was found between blood TG levels and liver lipid content in animals with acetonemia, a claim supported by other studies (McCarthy et al. 2015). In contrast, Sevinç et al. (2003) reported a significant decrease in total cholesterol, HDL-C and a slight decrease in TG, with no change in values of LDL-C in cows with hyperketonemia.

High levels of TG in the liver impair gluconeogenesis (Choi 2023). Their accumulation is associated with the development of hepatic steatosis in the transitional period, and in cows after calving with the development of SCK and CK, also found by Lean et al. (2013). Deposition of TG in the liver prevents the recovery of blood glucose concentrations and thus further prolongs lipolysis. The liver of the ruminants has a limited ability to export TG in the form of low-density lipoprotein (VLDL) (Grummer 1995), so limiting lipolysis is a successful transition from pregnancy to lactation. With the development of ketosis, an increase in the levels of TG, NEFA and BHBA in the blood, a decrease in the content of glycogen in the liver (90%), a decrease in gluconeogenesis (60%) and the oxidation of glucose (47%) were found (McCarthy et al. 2015). This indicates that cows lose glycogen during the first 14 days of lactation and have a reduced ability to utilize NEFA entering the liver (Ospina et al. 2010). Intensive fat mobilization and high energy requirements predispose animals to fatty liver dystrophy and ketosis. The occurrence of steatosis leads to a reduction in liver function and changes in the blood concentrations of glucose, total protein, cholesterol, TG, total bilirubin and urea (Djoković et al. 2013).

Our studies of the parameters of the mineral profile in the serum of cows of different physiological states (pregnant, recently calved and lactating) with SCK and CK found a decrease in the value of calcium, phosphorus and magnesium, reaching below 2 mmol/l Ca (hypocalcemia), below 1 mmol/l P (hypophosphatemia) and 0.5 mmol/l Mg (hypomagnesemia). The decrease in Ca and P values may be due to anorexia at the end of pregnancy and parturition, altering the balance between absorbable calcium and that secreted in milk. The beginning of lactation is a period that tests the ability of animals to maintain normal values of calcium, phosphorus and magnesium in the blood (Liesegang et al. 2007). Milk and colostrum are rich in these macroelements, which is why recently

calved and lactating cows must quickly adapt and regulate the losses that have occurred. On the other hand, increased gluconeogenesis and ketogenesis increase the need for phosphorus (Oetzel 2013). Another author (Fatur 1997) reported high levels of phosphorus in dairy cows with ketosis. Roche et al. (2015) found a positive relationship between BCS and calcium levels after calving, as well as a positive correlation between Ca and glucose values, in blood before and after parturition. Hypocalcemia in cows with CK is accompanied by high alakaline phosphatase activity (probably of bone origin), which is associated with increased mobilization of calcium from body stores (Sahinduran et al. 2010), also confirmed in our research. Kaya et al. (2016) reported low magnesium and P levels in cows with CK. Our results are consistent with these claims. During pregnancy, the need for minerals to form the skeleton of the fetus is also a cause of hypomineral states. These interpretations are supported by the research of other authors (Lean et al. 2013).

Conclusion

Early detection of the ketosis at the herd level by measuring BHBA and NEFA needs to become a mandatory preventive measure. Blood biochemical analysis of lipid profile in cows with SCK and CK demonstrated statistically significant changes-decreased levels of total cholesterol and HDL-C, while the blood levels of LDL-C and TG were increased. In cows with hyperketonemia, changes in the mineral profile are also observed - hypocalcemia, hypophosphatemia and hypomagnesemia. To prevent ketosis and its negative effect, it is necessary to develop strategies focused on the nutritional management of dry and transition cow by adjusting the ration and supplementing it with additional energy and mineral sources, so as to minimize the reduced food intake.

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