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Effects of Subclinical Ketosis and Subclinical Hypocalcemia on Some Biochemical Parameters and Reproduction in Cows

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ABSTRACT

Background: The purpose of the study was to evaluate the effects of Subclinical Hypocalcemia (SCH) and Subclinical Ketosis (SCK) diagnosed on the 10th day of lactation on reproductive performance.

Methods: The study used 120 clinically healthy Holstein cows aged 2 to 6 years who had just given birth and had no puerperal illness. Blood samples were collected on the 10th and 30th postpartum days and centrifuged at 3000 rpm for 15 minutes. The serum was removed and stored at -80°C until analysis. β-hydroxybutyrate (BHB), Non-esterified fatty acids (NEFA), glucose, cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), calcium (Ca), phosphorus (P) and magnesium (Mg) levels were measured. Cows with serum BHB levels >1.2 mmol/L were rated as SCK (n=10), those with serum BHB levels <1.2 mmol/L were rated as normal (n=50), those with serum total Ca levels <8.6 mg/dL were rated as SCH (n=30) and those with serum total Ca levels >8.6 mg/dL were rated as normal. Progesterone levels were evaluated using blood samples collected on the 30th day after parturition. To assess the reproductive performance of the animals in the study, the calving to first estrus, calving to first insemination and calving to conception periods for each animal were measured in days and the number of inseminations per pregnancy was estimated.

Result: In this study, no significant changes were observed in the biochemical parameters of cows with SCH. Calving to first estrus and calving to first insemination times were found to be higher in cows with SCH than in normal cows. There was no difference in other biochemical markers between normal and SCK cows, however NEFA and BHB levels were higher. While progesterone levels were higher in SCK-affected cows, there was no difference in reproductive parameters between these cows and non-ketotic cows.

Key words: Cow, Fertility, Subclinical hypocalcemia, Subclinical ketosis.

INTRODUCTION

In dairy cows, the transition period covers three weeks before and three weeks after calving. Complex metabolic adaptation processes take place during this time, including the transition from the dry period to milk production. However, adverse changes such as poor metabolic adaptations, decreased feed intake, increased insulin resistance, metabolic inflammation and diminished immune function may also occur during this time (Bradford *et al*., 2015; Grummer, 1995; Hammon *et al*., 2006). In cows, feed intake drops by 30% to 35% in the last three weeks before calving, resulting in a Negative Energy Balance (NEB). The severity of NEB increases immediately after calving (Grummer, 1995).

In recent years, advances in animal nutrition science have reduced macro-mineral imbalances, which may cause clinical symptoms in dairy cows. However, because hormonal and metabolic changes associated with the dry period and the onset of lactation can disrupt macro-mineral homeostasis, subclinical diseases can have a severe impact on dairy cow health and performance. Subclinical hypocalcemia (SCH) is one of these disorders (Reinhardt *et al*., 2011; Neves *et al*., 2016) and is described as a reduction in blood calcium concentration without clinical symptoms (such as lying down, lethargy, hypothermia and rumen atony). SCH is a macro-mineral metabolic disorder that occurs in cows in the transition period due to the disruption of Ca homeostasis (Xiao *et al*., 2019; Seifi *et al*., 2007). Various cut-off values varying between 8.0 mg/dL and 8.8 mg/dL are used to define subclinical hypocalcemia (DeGaris and Lean, 2008; Goff, 2008; Chapinal *et al*., 2011; Reinhardt *et al*., 2011; Martinez *et al*., 2012). Although clinical hypocalcemia is more severe, subclinical cases are more important because they occur more frequently, are difficult to diagnose and can have a negative impact on the cow's life expectancy and production (Goff, 2008; Murray *et al*., 2008).

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Ketosis is a common metabolic disease caused by NEB. NEB production causes an increase in NEFA and BHB levels in the blood. Depending on the elevated level of BHB, either clinical or subclinical ketosis develops (Duffield, 2000). Subclinical ketosis (SCK) is defined as an increase in ketone bodies (acetoacetate, acetone and β-hydroxybutyrate) in body fluids (blood, urine, milk) without symptoms of clinical ketosis (Andersson, 1988). BHB is the most common ketone body used to diagnose subclinical ketosis in dairy cows (Oetzel, 2004). BHB values of ≥1.2 to 1.4 mmol/L are considered SCK (Suthar *et al*., 2013). The cut-off value for BHB in subclinical ketosis has been reported as 1.2 mmol/L in the literature (Suthar *et al*., 2013; Belay *et al*., 2017; Ruoff *et al*., 2017; Chandler *et al*., 2018). Subclinical ketosis is associated with postpartum diseases, herd separation, decreased milk yield and poor reproductive function, resulting in significant economic losses. Consequently, in modern high-yielding dairy herds, it is a prevalent and significant metabolic disease (Duffield, 2000; LeBlanc, 2010).

In dairy cows, SCH has been reported to delay the resumption of cyclic activity of the ovary, prolong voluntary waiting time and negatively affect reproductive parameters (Caixeta *et al*., 2017; Seely and McArt, 2023). SCK has been linked to an increase in reproductive diseases and lower reproductive effectiveness, as well as a longer interval calving to conception (McArt *et al*., 2012; Shin *et al*., 2015; Ruterford *et al*., 2016).

Although subclinical hypocalcemia and subclinical ketosis in early lactation are known to negatively affect reproductive performance in dairy cows, the long-term effects of these two conditions on reproductive performance are not yet clear. As a result, this study aimed to evaluate the effects of SCH and SCK determined in the postpartum period on reproductive performance.

MATERIALS AND METHODS

Animal material

The research samples were collected from a farm in Tekirdağ province in 2023 and evaluated at Tekirdağ Namık Kemal University Faculty of Veterinary Medicine. The study included 120 (n=120) clinically healthy Holstein cows that had just given birth, were free of puerperal diseases (retained placenta, endometritis, laminitis and abomasum displacement), were between 2 and 6 years old, had an average daily milk yield of 35 kg. The study was approved by Tekirdağ Namık Kemal University's Animal Experiments Local Ethics Committee (date: 08.02.2023, number: T2023-1368). All animals were housed in free-range barns with the same level of care, feeding and reproductive control and were fed ad libitum with transitional Total Mixed Rations (TMR). Drinking water was clean and always accessible. Every cow received the same TMR twice a day and was automatically milked three times a day using a Delaval system. Following the guidelines outlined in AOAC (AOAC, 2002), the content and nutrient composition of TMR were measured in the Animal Nutrition and Nutritional Diseases laboratory of the Balıkesir University Faculty of Veterinary Medicine (Table 1).

Collection and processing of samples

Blood samples were collected on day 10 and 30 after birth from the vena coccygea using a 20-gauge vacutainer needle and placed in a 10 mL evacuated tube with no anticoagulant. Blood samples were centrifuged at 3000 rpm for 15 minutes to separate the serum and then stored briefly at -80°C before evaluation. An autoanalyzer (Randox, RX imola. Crumlin, United Kingdom) was used to evaluate BHB, NEFA, glucose, cholesterol, triglycerides, AST, ALT, Ca, P and Mg from blood samples obtained on the tenth day postpartum. Cows with serum BHB levels >1.2 mmol/L were rated as SCK (n=10), cows with serum BHB levels <1.2 mmol/L were rated as normal (n=50), cows with serum total Ca levels <8.6 mg/dL were rated as SCH (n=30) and cows with serum total Ca levels >8.6 mg/dL were rated as normal (n = 30). Progesterone values were determined by the ACS technique in the Automatic Chemiluminescence System (Siemens Immulite 2000) using ACS analysis kits in a special laboratory from blood samples taken on the 30th day postpartum. The voluntary waiting period lasted until the 45th day after calving. After day 45, increased cow activity determined by pedometer, increased uterine tone determined by rectal examination and the presence of preovulatory follicles indicated first estrus. The reproductive performance of the cows included in the study was monitored daily using farm records. Interval of calving to 1st AI and interval of calving to conception periods were recorded in days, as well as the number of inseminations per pregnancy.

Statistical analysis

The Shapiro-Wilk test was used to analyze the data's normality and evaluate whether it conformed to normal distribution. While the parametric independent sample t-test was used to examine the normally distributed data in the SCH and SCK groups, the nonparametric Mann-Whitney U test was used for the data that did not show a normal distribution. These procedures were performed using the GraphPad Prism 9.4.1 package application. Statistical significance was defined as $p<0.01$ and $p<0.05$.

RESULTS AND DISCUSSION

Cows with SCH had significantly decreased serum Ca levels ($p<0.01$) compared to normal cows. Other biochemical

Table 1: TMR nutrient composition (% DM).

DM% (dry matter)	58.33
CP% (crude protein)	18.44
CF% (crude fat)	4.44
CC% (crude cellulose)	13.37
C% (crude ash)	7.02
Starch%	26.73

indicators, however, did not show a significant difference between the two groups (Table 2).

Cows with SCK had considerably lower serum BHB levels ($p<0.01$) than normal cows, but no significant difference was observed in other biochemical parameters (Table 2).

Cows with SCH had significantly longer interval of calving to first estrus and Interval of calving to 1st AI than normal cows ($p<0.05$). Other reproductive parameters including progesterone values were found to be higher in cows with SCH, although the difference was not statistically significant ($p>0.05$) (Table 2 and Table 3).

There was no significant difference between cows with SCK and normal cows in terms of reproductive parameters (Table 3). On the other hand, progesterone value was found to be significantly higher in cows with SCK compared to normal cows ($p<0.05$) (Table 2).

The aim of the presented paper was to evaluate the effects of SCH and SCK, diagnosed on the 10th day of lactation on reproductive performance during 300 days of lactation. Additionally, the effects of SCH and SCK on some parameters were studied. Subclinical hypocalcemia has been defined with cut-off values ranging from 8.0 to 8.8 mg/dL (DeGaris and Lean, 2008; Goff, 2008; Chapinal *et al*., 2011; Reinhardt *et al*., 2011; Martinez *et al*., 2012). The cut-off value in this study was 8.6 mg/dL. Blood Ca level in animals with SCH (7.75±0.11 mg/dL) was significantly lower than in normal cows (9.32±0.15 mg/dL). The incidence of SCH in the animals in the study was found to be 50%, which is consistent with the studies by Reinhardt *et al*. (2011) and Chamberlin *et al*. (2013).

In the present study, serum P level was measured as 6.22±0.18 mg/dL in cows with SCH and 6.05±0.29 mg/dL in healthy cows. No difference was observed between the two groups in terms of P levels. P values were found to be compatible with the normal values (4-7 mg/dL) reported by Cheita and Sarma (2017). Patel *et al*. (2021) reported that there was no difference between the serum Mg levels of cows with SCH (2.35±0.09 mg/dL) and normal cows (2.17±007 mg/dL). As reported by Patel *et al*. (2021) and Hodnett *et al*. (1992) no difference was found between Mg levels of cows with SCH and healthy cows in this study. The reason for this may be that Mg deficiency is not always seen in SCH although it plays a role in hypocalcemia (Thilsing-Hansen *et al*., 2002).

Cai *et al*. (2018) and Reinhardt *et al*. (2011) reported that NEB developed in cows with SCH due to high milk yield and as a result, glucose levels decreased while NEFA and BHB levels increased. Mayasari *et al*. (2019) found no difference in glucose concentrations between cows with SCH and healthy cows. In the current study, no difference was found between the glucose, NEFA and BHB concentrations of cows with SCH and healthy cows, as reported by Mayasari *et al*. (2019). The differences between studies could be related to variations in the animals' daily milk yields.

Table 2: Comparison of biochemical parameters according to groups.

	SCH n=30	N n=30	p	SCK n=10	N n=50	p
Ca (mg/dL)	7.75±0.11	9.32±0.15	<0.0001	8.63±0.34	8.72±0.16	0.6100
P (mg/dL)	6.22±0.18	6.05±0.29	0.3693	6.61±0.37	6.18±0.18	0.1474
Mg (mg/dL)	2.19±0.05	2.12±0.09	0.4917	2.21±0.09	2.16±0.06	0.7288
Glucose (mg/dL)	57.18±0.98	57.71±1.42	0.7586	58.33±2.24	57.19±0.97	0.6347
BHB (mmol/L)	0.72±0.09	0.88±0.20	0.9409	2.03±0.22	0.54±0.03	<0.0001
NEFA (mmol/L)	0.36±0.03	0.41±0.09	0.5562	0.65±0.16	0.33±0.02	0.0136
Cholesterol (mg/dL)	116.60±9.25	127.00±12.85	0.4439	158.80±22.68	107.20±6.84	0.0259
Triglyceride (mg/dL)	9.93±0.76	10.48±1.40	0.7071	11.99±1.68	8.84±0.70	0.0778
ALT (U/L)	48.90±2.84	46.06±4.53	0.5809	50.00±3.42	46.80±2.73	0.6163
AST (U/L)	130.60±1.88	130.20±1.93	0.6265	132.9±1.32	130.20±1.80	>0.999
Progesterone (ng/mL)	0.48±0.24	0.068±0.01	0.4340	0.423±0.38	0.357±0.19	0.0167

Explanations: P<0.05; P<0.01; Ca- Calcium; P- Phosphorus; Mg- Magnesium; BHB- Betahydroxybutyrate; NEFA- Non-esterified fatty acids; ALT- Alanine aminotransferase; AST- Aspartate aminotransferase.

Table 3: Comparison of reproductive parameters according to groups.

	SCH	N	p	SCK	N	p
Interval calving to estrus (days)	122.00±9.71	96.55±19.17	0.0346	112.70±18.70	112.50±13.27	0.9948
Interval calving to 1st AI (days)	122.00±9.71	96.55±19.17	0.0346	112.70±18.70	112.50±13.27	0.9948
Interval calving to conception (days)	149.90±16.37	114.60±20.85	0.1175	148.70±30.26	136.70±16.55	0.7238
The number of inseminations per pregnancy (number)	1.73±0.18	1.55±0.16	0.05663	2.00±0.45	1.72±0.16	0.5674

Explanations: P<0.05.

Chamberlin *et al*. (2013) reported that AST levels did not differ between cows with and without SCH. In contrast, Ma *et al*. (2022) reported that cows with SCH had higher AST levels compared to healthy cows. According to Zaboot *et al*. (2017), AST levels did not change in the postpartum period, but increased due to morphological changes and muscle injuries during calving. Ma *et al*. (2022) reported that there was no difference between the triglyceride and cholesterol levels of cows with and without SCH. In our study, no difference was found between AST, ALT, cholesterol and triglyceride concentrations of cows with and without SCH. The reason for this could be due to differences in the timing of hypocalcemia diagnosis.

In dairy cows, there is a significant correlation between blood calcium levels and serum progesterone concentrations; postpartum cows with elevated blood calcium levels also exhibit elevated serum progesterone levels (Shoukat *et al*., 2022). However, in the present study, there was no significant difference in progesterone levels between cows with and without SCH. Further research on this subject is needed.

Early postpartum SCH (postpartum day 4) in multiparous cows has been shown to negatively affect reproductive parameters (Sedo *et al*., 2018). Furthermore, Caixeta *et al*. (2017) found that subclinical hypocalcemia lowers the pregnancy rate at first insemination and has an adverse effect on the restoration of ovarian function in cows during the voluntary waiting period. According to Sedo *et al*. (2018), SCH reduces the probability of pregnancy in dairy cows and prolongs the interval calving to conception. The current study indicated that cows with SCH had higher calving-first estrus, calving-first insemination, calving-re-conception times and the number of inseminations per pregnancy than healthy cows. These results are similar to the findings of Seely and McArt (2023); Sedo *et al*. (2018) and Caixeta *et al*. (2017). The prolonged interval calving to first estrus in cows with SCH may be explained by delayed ovarian activity due to decreased blood Ca levels (Jonsson *et al*., 1999). However, the mechanisms by which calcium delays ovarian activity have not yet been defined. The extension of the interval calving to conception can be explained by the increase in the uterine involution time and the increase in the return to cycle time previously reported in cows with SCH (Jonsson *et al*., 1999). It is believed that Ca deficiency occurring in SCH delays uterine involution by suppressing contraction of uterine smooth muscles.

The measurement of blood BHB level is considered the gold standard test for ketosis since it is more stable in blood than acetone and acetoacetate. For subclinical ketosis, the most widely used cut-off values are 1.2 mmol/L or 1.4 mmol/L for blood BHB (Dokovic *et al*., 2019). As described in the majority of the literature (Suthar *et al*., 2013; Belay *et al*., 2017; Ruoff *et al*., 2017; Chandler *et al*., 2018), the threshold value of BHBA for assessing SCK was taken as 1.2 mmol/L in our study. The blood BHB level was observed to be substantially higher in SCK-affected cows (2.03±0.22 mmol/L) than in healthy cows (0.54±0.03 mmol/L). In a study including cows from ten different European nations, Suthar *et al*. (2013) found that Turkey had the lowest frequency of SCK at 11.2% while Italy had the greatest prevalence at 36.6%. The incidence rate of SCK in the current study was assessed to be 16.6%, which was consistent with Suthar *et al*. (2013).

Ketosis is a common metabolic disease caused by NEB during the transition period in cows. This condition is characterized by relatively high concentrations of NEFA and ketone bodies in the blood while having low blood glucose concentrations (Grummer, 1995; Melendez *et al*., 2006; Shridhar, 2009; Dokovic *et al*., 2019). Li *et al*. (2016) found that cows with SCK had low glucose levels and high NEFA levels when compared to non-ketotic cows. In the present study, there was no difference in glucose levels between cows with SCK and non-ketotic cows, but NEFA levels were found to be significantly higher. High NEFA levels in cows with SCK indicate NEB and fat mobilization.

Antanaitis *et al*. (2019) stated that Ca and P levels did not change, while Mg levels decreased in cows with SCK compared to healthy cows. Yameogo *et al*. (2008) found that Ca and Mg levels decreased while P levels remained same in cows with SCK compared to healthy cows and they attributed this decline in Ca and Mg to a decrease in feed intake. Singh *et al*. (2021) also reported significantly lower Ca levels in cows with ketosis. The present study found no difference in Ca, P, or Mg levels between cows with and without SCK. The variations across the studies could be attributed to variances in mineral intake with ration.

ALT and AST are strongly associated with liver metabolism and are frequently used to assess liver function. When liver cells are damaged, ALT and AST are released into the bloodstream, leading to increased enzyme activity. It has been shown that SCK increased AST and ALT activity in the blood of dairy cows (Seifi *et al*., 2007; Mohsin *et al*., 2022). Similar to these studies, AST and ALT levels were higher in SCK cows than in healthy cows in this study, however this was not statistically significant. Ha *et al*. (2022) found no difference in the triglyceride and cholesterol levels of cows with and without SCK. Similarly, there was no difference between the two groups in terms of triglyceride and cholesterol concentrations in our study.

It has been shown that in NEB cows with high BHB levels, insulin-like growth factor (IGF) secretion is reduced, follicular development and estradiol secretion are reduced (Pishvaei *et al*., 2021). However, the effect of NEB and SCK on progesterone is not yet clear. In this study, progesterone levels in SCK cows were significantly higher than in normal cows. However, although higher in SCK cows than in normal cows, they were not above the 1ng/mL threshold for progesterone, so further studies with repeated progesterone measurements are needed on this topic.

SCK has been linked to higher reproductive issues and lower reproductive performance (Shin *et al*., 2015). It has been found that in cows with SCK, the interval calving to first estrus, interval calving to 1st AI and interval calving to

conception is extended (Rutherford *et al*., 2016). Decreased reproductive performance has been linked to a delay in the estrus cycle caused by a decrease in GnRH and LH production, which are required for follicular development and ovulation (Butler, 2003). At the same time, reproductive efficiency is highly correlated with energy availability and may be affected by energy deficits in early lactation. Rodriguez *et al*. (2022) reported that hyperketonemia diagnosed in the first week of lactation was negatively associated with reproductive performance, whereas hyperketonemia diagnosed in the second week of lactation was not negatively associated with reproductive performance. In the present study, SCK was diagnosed on the 10th day of lactation and there was no difference in reproductive parameters between cows with and without SCK. These results were consistent with Rodriguez *et al*. (2022).

CONCLUSION

The incidence of SCH in the present study was found to be 50%. The fact that the interval of calving to first estrus and 1st AI in cows with SCH were found to be higher than in normal cows, with negatively affects long-term reproductive performance. The incidence of SCK was determined as 16.6%. It was found that in cows, SCK affected only biochemical markers of BHB and NEFA, with no impact on other markers. High NEFA levels in cows with SCK were indicative of NEB and fat mobilization. Although there was no difference between the reproductive parameters of cows with SCK and normal cows, progesterone levels were found to be high in cows with SCK. However, further research is needed to better understand the effects of SCH and SCK on reproductive performance in dairy cows.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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